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# Adenovirus Evasion of Cell-Intrinsic Immunity

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LOYOLA UNIVERSITY CHICAGO

ADENOVIRUS EVASION OF CELL-INTRINSIC IMMUNITY

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THE FACULTY OF THE GRADUATE SCHOOL  
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DOCTOR OF PHILOSOPHY

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BY

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## ABSTRACT

Virus cell entry represents one of the earliest opportunities for a host to respond to infection. Understanding the processes of pathogen detection and restriction employed by the host, as well as strategies utilized by the virus itself to evade such processes, is critical in developing therapeutics to counter pathogenesis. Adenovirus (Ad) infections are self-limiting in healthy populations, but can be devastating to individuals with compromised immune systems. Currently, no specific antiviral treatments exist to combat Ad infections in susceptible populations. However, because Ad infections are not severe in healthy individuals, employing replication-defective Ads as vaccine vectors is generally regarded as safe, and as such are undergoing thorough investigation. The effects of Ads on human health, both from infection and as a vaccine vector, are substantial; however there are still informational gaps when describing the virus' interactions within host cells to produce an infection. Furthermore, viruses manipulate specific pathways to ensure they productively replicate and produce progeny virions, making them ideal tools to probe such complex host pathways.

Our lab uses Ad to investigate both mechanisms of host-mediated restriction of virus and the pathogen's methods of evading detection. Understanding the contributions of distinct mechanisms employed by Ad to evade host responses and efficiently enter cells will enhance the efficacy of future Ad vaccine vectors and identify novel targets for antiviral drug developments. Previous studies in our lab show that Ad-induced membrane

rupture stimulates a host cell response characterized by the recruitment of autophagy machinery to sites of damage. Other observations suggest that wild-type Ad5 virions evade sequestration by autophagy via the recruitment and engagement of microtubule motor proteins. Current studies suggest that multiple viral proteins making up the viral capsid function to aid in motor protein recruitment and viral transport throughout the cell, but the specific details of these molecular interactions have not been entirely discerned. This study examines the mechanisms of autophagy induction by the host during Ad entry, and assesses the influences of capsid proteins of virion engagement with microtubules to facilitate endosomal egress and translocation to the nucleus.

## CHAPTER I

### INTRODUCTION

#### **Virus-host interactions**

To survive and proliferate, intracellular pathogens, such as viruses, must evade and subvert numerous host cell defenses and establish an infection. To do this, viruses overcome passive barriers, such as crossing the cell membrane or trafficking to sites of replication, either through direct action or by exploiting host machinery. At the same time, once the cell is alerted to the pathogen, the host employs active defenses to curtail infection, posing a major barrier to pathogenesis. These defenses include innate responses, which attempt to degrade incoming viruses, and adaptive responses to recruit immune cells, limit the infection from spreading, and suppress recurring infections. Different viruses have evolved various means to overcome both passive and active barriers, either through use of viral capsid proteins or with the generation of proteins *de novo* during replication. The ability of the virus to subvert these processes is essential for pathogenesis. Many of these active defense mechanisms are typically general cell processes utilized in times of stress, and as such have been characterized and studied in the context of the general cell life cycle. However, how pathogens initiate, and subsequently counteract host-mediated restrictions limitations are not fully understood. Further elucidation of the induction and maintenance of host cell defense mechanisms

during pathogenesis, and assessing how pathogens evolve to disrupt such limitations, will aid in the development of therapeutics against disease.

### *Intrinsic innate immunity*

The first response against the virus after entry is initiated occurs within the infected cell itself. This response requires an immediate and direct response, called “intrinsic innate immunity,” which defines factors that immediately recognize viral components and restrict infection and/or replication [1]. This response consists of cytosolic pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) and RIG-I-like receptors (RLRs), as well as host restriction factors that are either constitutively expressed within the cell at basal levels, or proteins whose expression is upregulated relatively quickly through IFN signaling.

Some examples of intrinsic innate immunity factors include IFN-inducible transmembrane proteins (IFITMs) that restrict influenza virus [2, 3] and the myriad of factors that attempt to restrict HIV uncoating and reverse transcription during entry, such as TRIM5 $\alpha$  [4] and APOBEC3G [5]. Other factors such as cGAS and DDX41 sense incoming foreign DNA, such as viral genomes, and activate STING-dependent signaling cascades to produce interferons [6-11]. Defensins also inhibit virus infection by restricting their entry, yielding yet another pathway hosts utilize to mediate infection [12-14]. Viruses often utilize varied strategies to evade these host elements, establish an infection, and replicate. The presence, or indeed absence, of certain factors in some cell types may in part determine the permissiveness of a cell to succumb to viral infection. Co-evolution of a virus with its host counterpart places immense positive selection

pressure on both organisms to best each other, in a sort of virus-host “arms race.” For these reasons, studying the mechanisms by which these factors are activated and subsequently recognize and restrict virus entry is critical for the future development of antivirals. In depth studies of both the host factors or complexes that restrict viruses, as well as the viral proteins critical for evading these responses, will not only shed light on the complex interactions between the two, but may also further investigations into parallel host-virus interactions that currently elude us.

#### *Innate immune responses to pathogen infection*

In addition to immediate response factors, the host cell contains a multitude of sensors to detect non-self components expressed by pathogens. Activation of these sensors results in stimulating an equally complex set of signaling pathways, and ultimately changes in host protein expression or function both within the infected cell, and also alerts neighboring cells to the presence of a pathogen. Host PRRs recognize both pathogen-associated molecular patterns (PAMPs) from infectious agents as well as conserved danger molecules produced by the cell in times of stress (danger-associated molecular patterns, or DAMPs). These unique patterns, though structurally diverse, are conserved across many pathogens, and require differential recognition by PRRs via distinct ligand-recognizing domains. PRRs are characterized by structure, specificity, cellular localization, and tissue-specific expression. Currently identified PRRs are grouped into multiple classes, including membrane-localized TLRs, RLRs, C-type lectin receptors (CLRs), intracellular NOD-like receptors (NLRs), and cytosolic nucleic acid sensors such as the Pyrin-HIN domain-containing family, OAS proteins, and cGAS [15-

20]. There are also soluble, secreted PRRs, such as oligosaccharide-binding collectins, that recognize moieties on microbial cell surfaces and allow macrophages and neutrophils to phagocytose them.

### *Interferon signaling*

Innate detection of incoming virions at the cell surface or within the cytoplasm by PRRs initiates downstream pro-inflammatory effectors that consequently put neighboring immune cells – including macrophages, monocytes, neutrophils, and NK cells – in an alerted antiviral state within hours of the initial infection [21, 22]. For example, several TLRs present on the cell surface and within endosomal membranes recognize viral moieties, eliciting signals that activate the transcription factors IRF3 and NF- $\kappa$ B to enhance Type I interferon (IFN) expression [22, 23]. Produced IFNs are secreted and act as ligands for neighboring cells, subsequently inducing an antiviral state in the nearby cells through the induction of antiviral interferon-stimulated genes (ISGs), as well as contributing to an adaptive immune response [24]. There are three known classes of interferons: type I IFNs consist of IFN- $\alpha$  and IFN- $\beta$ , the type II IFN class has only one member (IFN- $\gamma$ ), and type III IFNs contain of three distinct molecules of IFN- $\lambda$  [25]. Type I and type III interferons have predominant roles during viral infections, and are produced by nearly every cell type upon recognition of a viral moiety [26-28].

### *Proinflammatory Responses*

In addition to IFN production, viral penetration into cells may result in the production of proinflammatory cytokines, such as IL-1 $\beta$ , TNF- $\alpha$ , and IL-6. These proinflammatory cytokines activate an antiviral response and recruit other immune cells

such as macrophages and neutrophils. A class of NLRs recognize virus and oligomerize into a structure called the inflammasome, which associates with caspase-1 to cleave it into its active form. Active caspase-1 subsequently cleaves pro-IL-1 $\beta$  and pro-IL-18 into mature IL-1 $\beta$  and IL-18, facilitating their secretion [29-31]. Studies suggest that reactive oxygen species (ROS) produced upon oxidative stress within the cell is an important factor during inflammasome activation [32]. Protein kinase R (PKR), a host molecule that recognizes viral RNA in cells, is implicated in positively regulating NLRP3 inflammasome activation, though the mechanism is unknown [33-35]. In addition to inflammasome formation, NLRs have a myriad range of functions during pathogen infection. It activates a number of signaling pathways, including p38 mitogen-activated protein kinase (MAPK) and ERK stimulation, which activate NF- $\kappa$ B-dependent transcription to produce inflammatory chemokines such as CXCL10 and CCL5 [36-38]. These chemokines are important to recruit immune cells to sites of inflammation, initiating the adaptive immunity arm of the immune response. NLRP3 inflammasomes also have the capacity to initiate cell death through pyroptosis as a sort of “self-destruct” if the cell is succumbing to microbial infection [39].

#### *Adaptive immunity*

Well-established literature indicates that PRR signaling and inflammasome activation are critical factors in controlling the development of the adaptive immune response upon intracellular pathogen exposure. After the initial response to infection, the production of IFNs and inflammatory cytokines and chemokines stimulate and recruit adaptive cells to the site of infection, and direct an immune response that is highly



specific towards the infecting pathogen [40]. While the explicit roles of specific classes of PRRs in inducing the adaptive immune response are still not entirely clear, current data indicate that PRR-mediated signaling guides the specific actions induced during the adaptive response. For example, PRR signaling in dendritic cells (DCs) governs the particular antigens presented to T and B cells, as well as help to dictate the effector proteins produced by lymphocytes during infection [41]. The combinations of recognition by individual innate immune molecules and how effector cells of the adaptive immune system interpret the given signals are key factors in generating the appropriate type of effector response.

Pathogen recognition and signaling by DCs also play a clear role in effector T cell maturation, as mouse models lacking specific populations of functionally distinct DC subsets generate varying T cell responses [42-44]. Indeed, direct PRR activation within a DC subset is required to activate specific helper T cell responses [45]. Resident antigen presenting cells (such as DCs) take up antigen and display antigenic epitopes MHC class II molecules to the local lymphatic system [46]. Antigen presentation to naïve  $CD4^+$  T cells in a lymph node engages the T cell receptor (TCR), and CD80/86/CD28 ligand/receptor costimulation on T cells, as well as IL-18 and IL-12 cytokine secretion skew lymphocyte differentiation to  $CD4^+$  T helper type 1 cell ( $T_H1$  cell) populations [40].  $T_H1$  cells produce  $IFN\gamma$  to facilitate antibody class switching in activated B cells to opsonizing IgG classes and support  $CD8^+$  T cell activation [47]. Other T helper cell populations are induced upon extracellular infection ( $T_H17$  cells) or B cell interactions and allergic responses ( $T_H2$  cells). Activated effector helper T cells have the capacity to

interact with B cells, providing necessary signals (both direct and indirect) to facilitate B cell proliferation and maturation into antibody-secreting plasma cells [48]. A small subset of these T cells also generate a population of memory cells that persist within the host long after the infection is cleared, and offer a source of rapid protection upon encountering the same antigen [49].

During intracellular pathogen infection, infected cells express antigenic epitopes loaded onto MHC class I molecules, which are expressed in virtually all nucleated cell types. CD8<sup>+</sup> cytotoxic T cells (CTLs) recognize antigens in the context of MHC class I molecules, and costimulation activates CTLs to facilitate killing of the infected cells [47]. CTL-mediated cell death is effected by T cell release of perforin- and granzyme-containing cytolytic granules, which lead to cell apoptosis. Recent reports suggest that in addition to cytolytic effects, a subset of memory CD8<sup>+</sup> T cells also have the ability to provide T cell help. Upon re-exposure to a pathogen, these cells produce IL-4 and other cytokines to support B cell migration to sites of infection, subsequently activate the B cells and facilitate antibody class switching [50, 51].

Many studies use viruses and other pathogens to study changes in the immune response during infection, with a substantial focus on adaptive responses and long-term immunity. However, current efforts have shifted to using certain viruses as tools to deliver transgenes into host cells for both gene therapy and vaccine development [52]. Years of co-evolution have developed viruses into operational nanomachines that are already extremely effective at delivering genetic material. Using viruses as a pre-existing platform for gene delivery requires a dynamic change in research focus: in addition to

studying broad adaptive host responses, also investigating more deeply the intrinsic innate responses against initial infection to allow optimal transgene delivery, and immune responses thereafter. Such a reassessment of each individual step of the immune response as a whole, as well as their interplay with each other to produce an optimal response against transgene expression, will allow researchers to develop very specific and targeted viral platforms as vaccine candidates. This dissertation will focus on some components of the initial host restriction of adenovirus (Ad), as well as viral mechanisms of evading such restrictions.

### **Autophagy as an innate antimicrobial response**

One particular intrinsic innate immune response is a regulatory mechanism called autophagy. Autophagy is a host process generally utilized by the cell to sequester excess or damaged cytoplasmic macromolecules and host organelles, typically to facilitate degradation of these complexes. Three main forms of autophagy occur within the cell: macroautophagy, microautophagy, and chaperone-mediated autophagy [53]. Macroautophagy (referred to as simply autophagy henceforth) is the most studied type, and forms a double-membraned vesicle, or autophagosome, around cytosolic contents to sequester them. Degradation of these contents occurs upon autophagosomal fusion with another vesicular compartment, the lysosome [53]. Low pH and high concentrations of hydrolytic proteases, sulfatases, and glycosidases break down lysosomal contents to provide free amino acids for *de novo* protein synthesis.

In addition to autophagy's necessity during the general cell cycle, it also serves a role as a defense mechanism against numerous intracellular pathogens (called

xenophagy). Cell sensing of pathogen invasion through different pathways ultimately elicits autophagosome formation to isolate and degrade pathogens [54]. This function is important for innate immune responses during viral infections, as degradation exposes the viral genome to TLRs, and DNA recognition leads to downstream production of Type I IFNs. Due to this fact, many pathogens have evolved means to inhibit or exploit autophagosome formation during infection. For example, multiple bacterial pathogens evade being targeted to autophagosomes by replicating undetected within vesicles, isolated from the cytoplasm and much of the autophagy-inducing machinery. Additionally, almost all known viruses utilize varied methods to circumvent restriction by autophagy. Some DNA viruses, such as HSV-1, HCMV, and KSHV all suppress autophagy activation almost entirely. Others, including EBV, SV40, VZV, and hepatitis B viruses induce autophagy during infection, but readily evade restriction [55]. On the other hand, many RNA viruses colocalize with autophagic membranes during replication [56], suggesting their utilization and exploitation of this host cell defense.

It has become clear that basal levels of autophagy are not sufficient to restrict intracellular pathogen infection, and as such research has recently begun to investigate the specific signals required to induce autophagosome formation. One major signaling pathway molecule involved in autophagy induction during bacterial infection is the kinase mammalian target of rapamycin (mTOR). In *Shigella*, *Listeria*, and *Salmonella* infections, membrane damage inflicted by these pathogens rapidly inhibits mTOR, enhancing autophagy induction. Increased autophagosome formation is persistent in the case of *Shigella*-induced damage, but both *Listeria*- and *Salmonella*-mediated rupture see

a transient increase [57-59]. Interestingly, the *Salmonella* pathogenicity island 1 (SPI-1) recruits the host kinase focal adhesion kinase (FAK) to activate mTOR activity [60]. This likely mediates the transient mTOR inhibition observed during *Salmonella* infection. Other, mTOR-independent autophagy induction pathways are also implicated in pathogen restriction. Beclin-1 complex activation through the PI3 kinase signaling pathway enhances autophagosome formation, as evidenced by multiple viruses incorporating genes that specifically regulate this complex [61-67]. These observations indicate that numerous pathways are in place to activate autophagosome-mediated restriction of many different pathogens. The fact that the majority of intracellular pathogens evolved mechanisms to evade this response is significant, implicating autophagy as a major player in restricting intracellular pathogen infection during cell entry.

How the host specifically recognizes and targets pathogens to autophagosomes is diverse, depending on the organism, and these various approaches are currently under investigation. Nod1 and Nod2 proteins recognize peptidoglycan moieties on bacterial pathogens contained within vesicles. This detection leads to downstream signaling events, activating NF- $\kappa$ B and mitogen-activated protein kinase (MAPK) pro-inflammatory pathways in a Rip2-dependent manner [68, 69]. Nod2 also interacts with the autophagy component ATG16L1 in a Rip-2-independent manner, recruiting it to bacterial-containing vacuoles near the plasma membrane [70]. While current data clearly implicate Nod proteins in targeting bacteria contained within vesicles, it is unclear whether they also have the ability to sense bacteria that have escaped into the cytosol.

In the case of pathogens requiring membrane rupture to access the cytosol, our lab and others suggest that galectin recruitment is an important nucleating event to induce autophagosomal targeting. Galectin-3 is reported to colocalize with the autophagosomal marker, LC3, after membrane rupture during *Shigella* infection [71]. Upon endosomal lysis by *Salmonella enterica*, galectins 3, 8, and 9 are all recruited to the site of injury, and of these three, siRNA knock down of only galectin-8 significantly limits LC3 recruitment to *Salmonella*-containing vacuoles and increases bacterial replication [72]. This report also showed that galectin-8 recruitment of NDP52 occurs more rapidly than ubiquitin-mediated recruitment. These studies suggest that galectin-8 binding damaged membranes facilitates a rapid recruitment of other factors important for autophagy induction.

Ubiquitin may also play a vital role in LC3 recruitment. Upon rupture, various proteins from both the host and the pathogen become polyubiquitinated by host proteins [72, 73]. The E3 ubiquitin ligase leucine-rich repeat and sterile alpha motif-containing 1 (LRSAM1) ubiquitinates *Salmonella* organisms, and this ligase's activity is necessary for recruiting NDP52 [74]. Knock down of LRSAM1 increases *Salmonella* replication within *Salmonella*-containing vacuoles (SCVs), but its function in other infection models has not been assessed. Furthermore, *Salmonella* de-ubiquitinating (DUB) activity interferes with autophagy, suggesting that ubiquitination is necessary for autophagy-mediated restriction [75, 76]. Data support that ubiquitin acts as part of a protein "bridge" to enhance LC3 membrane association with pathogens (discussed below). Inhibiting global protein ubiquitination by a chemical inhibitor of E1 ubiquitin activating enzymes severely

restricts LC3 recruitment to ruptured endocytic membranes during transfection of polystyrene beads, where, presumably, the transfection reagent is rupturing endosomes [77]. The authors argue that since the beads are not proteinaceous, and thus cannot be ubiquitinated, then it is host protein ubiquitination that recruits autophagy machinery. In live-cell imaging experiments, LC3 recruitment to ruptured membranes occurs up to 15 minutes after protein ubiquitination, and knocking down autophagy machinery has no effect on membrane fragment ubiquitination.

After ubiquitination, so-called adaptor proteins have the capacity to bind ubiquitin and subsequently recruit LC3, targeting ubiquitinated targets to initiating autophagosomes. At present, four proteins are identified as adaptor proteins: p62, NDP52, NBR1, and optineurin (OPTN) [78-81]. NDP52 binding to ubiquitinated molecules recruits a protein complex including the host kinase TANK-Binding Kinase-1 (TBK1). TBK1 phosphorylates p62 and OPTN to increase their affinities for LC3 after binding ubiquitin [81, 82]. It is unknown whether NBR1 is phosphorylated by TBK1. Preventing TBK1 function in MEFs either through siRNA knock downs or chemical inhibition increases the number of autophagosomes within the cell, but prevents their maturation and fusion with lysosomes [83]. This indicates that TBK1 function occurs after autophagy is induced, but is important in their maturation process. The importance of TBK1 in infections has precedence in both bacterial and viral contexts. TBK1 is a critical factor in limiting replication in a number of bacterial pathogens via autophagosomal maturation, and HIV accessory proteins Vpr and Vif block TBK1 autophosphorylation to inhibit the induction of both Type I and Type III interferons [82-

86]. These data suggest that TBK1 is critical not only for autophagosome sequestration of pathogens, but also plays a role in interferon production during infection. Interestingly a second host kinase, Unc-51-like kinase (ULK1), also phosphorylates p62 at S409 [87], but biochemical assays indicate that p62 phosphorylation by ULK1 inhibits TBK1-mediated phosphorylation on S403 [88]. Casein kinase 2 (CK2) also phosphorylates S403 [89], suggesting that CK2 phosphorylates p62 as an alternative to TBK1 in this case. It is noteworthy that both ULK1 and CK2 phosphorylation of p62 occurs through mTOR signaling. Perhaps TBK1-dependent phosphorylation of p62 is important in mTOR-independent autophagy induction pathways. Nevertheless, a number of reports clearly implicate the importance of TBK1 phosphorylating activity in retaining pathogens to autophagosomes.

## **Microtubules**

### *The microtubule network*

Microtubules (MTs) are one of three cytoskeletal networks in the cell, along with intermediate filaments (IFs) and microfilaments (actin) [90, 91]. MTs are a major component of trafficking various molecules throughout the cell, including protein complexes, cytoskeletal components, various membrane-bound organelles, and even pathogens. Cargo motilities on MTs are some of the fastest in the cell, and are composed of both short- and long-range, directional movements. They maintain cell shape, influence cell movement, and separate chromosomes during cell division. Their assembly is dynamic, consistently depolymerizing (catastrophe) and re-polymerizing (rescue). This



complex oscillation of short and long structures is important for reorganizing the cell, especially during mitosis.

MTs are tubular, helical rods consisting of dimers of globular proteins  $\alpha$ - and  $\beta$ -tubulin that extend from the microtubule organizing center (MTOC) near the nucleus throughout the cell cytoplasm [92]. A third form of tubulin,  $\gamma$ -tubulin, anchors MTs at the MTOC and initiates the polymerization of new MTs [93]. Dynamic instability of MTs allows the cell to quickly alter and modify the cell's architecture. MTs are polar structures, containing a minus end anchored at the MTOC, and a plus end that extends outward where nascent tubulin dimers are added. Catastrophe and rescue preferentially occur at MT plus ends.  $\alpha/\beta$ -tubulin dimers bound to GTP readily associate to MT plus ends, supporting MT growth. Rho GTPases hydrolyze GTP on MT-associated tubulin, diminishing its affinity to maintain an interaction with other tubulin molecules [94, 95]. Growth and/or destabilization of microtubules depends on both the rates of tubulin addition to MT plus ends as well as GTPase activity. As long as GTP-bound dimers are added at a faster rate than GTP hydrolysis occurs, microtubules retain a GTP-capped plus end, continuing growth. Once a critical concentration of GDP-bound tubulin is reached due to increased GTPase activity or decreased tubulin availability, the MT quickly depolymerizes, shrinking back toward the MTOC. Most MTs undergo rapid turnover, having a half-life of approximately a few minutes.

*Microtubule motor proteins facilitate transport throughout the cell*

Travel throughout the cell upon microtubules is mediated by two families of motor proteins: cytoplasmic dynein (hereafter referred to simply as dynein) complexes,

and protein complexes from the kinesin superfamily [96, 97]. With very few exceptions, dynein molecules traffic cargo in a retrograde fashion towards MT minus ends (i.e., the MTOC and nucleus), and kinesin complexes transport molecules anterogradely to the cell periphery, towards MT plus ends. Many kinesin motors exist, and are differentially expressed in humans based on cell type. However, current research suggests that only one gene exists for the dynein motor, expressed ubiquitously in all cell types [98]. Most cargoes simultaneously interact with dynein and kinesin motors, with directional transport towards the proper destination mediated by back-and-forth movements along MTs culminating in an overall net course [99]. This method of travel likely allows the motors to avoid obstructions in its path, such as overlapping MTs or MT-bound proteins in the way. Alternatively, it may provide a means around an all-or-nothing decision of material transport, whereby the cargo reaches its end point via numerous, small movements, grossly culminating towards the proper direction. The interplay between these two groups of motors is complex, and their functions are absolutely necessary for cell viability.

Dynein motors are a large (1-2 MDa) complex of proteins made up of a motor and various molecules that direct cargo specificity. The dynein heavy chain (DHC) is the central component of the complex, and contains the motor function of the complex at its C-terminus and an N-terminal tail domain, responsible for interacting with other complex components [100]. The motor domain is made up of a microtubule-interacting stalk and six AAA+ (ATPase associated with diverse cellular activities) domains, arranged in a ring-like structure. ATP hydrolysis generates force to move dynein and its cargo. The

mechanism of converting energy from ATP to ADP to generate such a force is not well understood, although recent advances suggest that ATP hydrolysis acts in concert with dynein attachment to, and detachment from, microtubules, coordinated by open and closed conformations of the AAA+ ring [101, 102].

A dimer of two DHCs makes up the core of the dynein complex. The tails of each dimer bind both two intermediate and two intermediate light chains (IC and LIC, respectively). IC and LIC genes contain multiple alternative splice sites, producing a range of tissue-specific isoforms that potentially enact different functions [103-105], including differential interactions with other dynein complex proteins [106]. Different isoforms of ICs localize dynein to varying organelles, and research suggests that neuronal cells discriminate dynein motors based on the specific variants of ICs present. Isoforms can also heterodimerize on dynein, potentially increasing potential cargo interactions for that motor. IC and LICs subsequently bind two of three distinct light chains (LCs): Tctex-1, LC8, and LC7/Roadblock. Additionally, dynein interacts with a number of regulators, including dynactin, Lis1, NudE, and NudE-related proteins Nde1 and Nde2. These regulators control a number of aspects of dynein function, including microtubule association during spindle assembly and cargo specificity. Dynactin in particular, which is itself a complex of proteins, is a cargo-binding subunit critical for normal dynein function. Point mutations in the dynactin subunit p150<sup>Glued</sup> cause a Parkinson-like disease, Perry syndrome [107].

Kinesins are a superfamily of proteins; 14 classes contain approximately 45 different genes. Similar to dynein, a dimer of kinesin heavy chains (KHCs) make up the

core of the complex, which also contain an ATP-hydrolyzing motor complex and protein-binding tail [98]. The motor domain is highly conserved among kinesins, but the tail is highly variable. Most kinesins move anterogradely along microtubules, but observations show that some types move towards the nucleus, and others even affect microtubule shrinking and growth. This study focuses on an isoform of a major kinesin molecule Kinesin-1, Kif5B. Interestingly, kinesin molecules maintain themselves in an auto-inhibited state when they are not transporting cargo, where the tail domain folds in over the motor head [108]. Interactions with cargo or phosphorylation by regulatory kinases unfold kinesin, activating its motor function. Evidence also implicates RanBP2 (also known as the nucleoporin Nup358) as an activator of Kif5B function [109, 110].

The structure of the kinesin complex is comparable to that of dynein: KHC molecules dimerize through coiled-coil interactions, and, via their tail domains, subsequently interact with two kinesin light chains (KLCs), which control cargo specificity. In humans, there are four distinct isoforms of KLC: KLC1, KLC2, KLC3, and KLC4 [111]. KLC1 and KLC2 are the most-studied light chains. They are ubiquitously expressed in various tissues, and share 87% sequence identity between their cargo-binding domains. KLC cargo binding depends on the interactions of proteins with tetratricopeptide repeat (TPR) domains, which are helical tandem repeat structures consisting of 42 amino acids. KLC1 contains six TPRs, while KLC2 totals 5 and one-half, leading to slightly different cargo specificities.

*Microtubule post-translational modifications*

Although the makeup of MTs seems simple, they are involved in a vast number of applications in the cell. To accomplish all of these functions, MTs are regulated by a number of mechanisms, most notably post-translational modifications (PTMs) [112, 113]. The multitude of PTMs that occur on MTs affect their dynamic stability, as well as the molecules they selectively recruit and bind. Some PTMs are ubiquitous, such as acetylation and phosphorylation, functioning as binary on/off switches. Others are potentially unique to microtubules, including tyrosination and glutamylation. PTMs have the capacity to occur concurrently, giving a vast complexity to the functions of individual MTs. Most PTMs, with the exception of acetylation, occur on the outside surface of the MTs, modifying the C-terminal tails of the tubulin molecules. In contrast, acetyltransferases modify the luminal surface of MTs.

Mouse cells deficient in acetylated MTs are more resistant to nocodazole treatment, and  $\beta$ -tubulin acetylation on its lysine residue at position 252 inhibits microtubule rescue, leading some to postulate that acetylation likely does not regulate the binding of motor proteins or other microtubule-associated proteins (MAPs), but regulates the assembly of nascent MTs [114, 115]. However, a more recent report indicates that acetylation enhances p38-MAPK activation in macrophages, and increases Hsp90 recruitment to MTs. The data suggest that Hsp90 binding activates Akt and p53 signaling [116]. Studies also show that both  $\alpha$ - and  $\beta$ -tubulin subunits are phosphorylated, however the exact function of this activity is unknown. Phosphorylating  $\beta$ -tubulin at residue S172 may play a role in regulating MT dynamics during cell division, but no function has been

attributed to  $\alpha$ -tubulin phosphorylation [117]. Other common PTMs seen on MTs include methylation, palmitoylation, ubiquitination, and sumoylation, although no functions involving these modifications have been revealed.

Microtubule tyrosination is the reversible act of appending a tyrosine residue to the C-terminal end of unpolymerized tubulin molecules [117, 118]. Tubulin sequences reveal that tyrosine is initially encoded within  $\alpha$ -tubulin, suggesting that tyrosine removal, or detyrosination, is the initial modification. The mechanism of detyrosination is yet to be revealed, but tyrosination occurs via the tubulin tyrosine ligase, or TTL. De/tyrosination activity is a critical function in development, as TTL-deficient mice die within 24 hours after birth. A number of functions are attributed to tubulin detyrosination, which leaves the penultimate glutamate residue as the terminal amino acid. It modulates the interactions of MTs with both various MAPs and other cytoskeletal filaments. Detyrosination also restricts MT turnover by negatively regulating the mitotic centromere-associated kinesin (MCAK), which rapidly depolymerizes microtubules [112]. It also reduces kinesin-1 processivity, but enhances kinesin-2 recruitment [119]. On the other hand, tyrosinated tubulin is important for spindle formation, implicating its importance in mitosis [112]. These differences in functions suggest that the addition and removal of tyrosine to the C-terminus of tubulin molecules is an essential regulator of microtubule activity and stability. In fact, another PTM that modifies the C-terminal tail of proteins is the removal of either one or two glutamate residues after detyrosination ( $\Delta 2$ -tubulin and  $\Delta 3$ -tubulin, respectively) [120]. Studies into these modifications are not fully fleshed out, but in rat neuronal cells they are indicative of very long-lived

microtubules [121]. These molecules cannot be re-tyrosinated after glutamate removal, indicating that at least one of the roles of glutamate removal is to enhance microtubule stability by preventing the addition of tyrosine.

Two other PTMs that occur on tubulin are polyglutamylation and polyamination, which add side chains of glutamates or amines, respectively, to gene-encoded glutamate residues [122, 123]. Chains are typically appended to C-terminal amino acids, and are hypothesized to regulate electrostatic interactions between MTs and associated proteins by changing the charge on the MT surface, although no experimental evidence yet supports this claim. Large glutamate chains (Glu<sub>10</sub>) appear to decrease kinesin-1 processivity, but shorter chains (Glu<sub>3</sub>) do not enact this effect [119]. Synthetic assemblies suggest that polyglutamylation also enhances dynein processivity, but the relevance of this in vivo has not been pursued. Additionally, polyglutamylation reduces MT stability while polyamination enhances it, especially in MT destabilizing conditions induced by cold or calcium-containing environments. Interestingly, while polyglutamylation is a reversible process, polyamination is likely irreversible [112, 113]. More research into these modifications is required to understand their specific contributions to microtubule function during the cell cycle.

#### *The endosomal pathway and microtubule trafficking*

MT networks are vital in sustaining the transport of endocytic vesicles throughout the cell. Every endosome actively moves throughout the cell on MTs, and transport is dependent on the type of endosome [124]. Extracellular contents are taken up by EEA1<sup>+</sup>/Rab5<sup>+</sup> early endosomes (EEs), which are formed at the plasma membrane,

generally in an actin-dependent manner [125]. The actin motor myosin VI, along with +TIP proteins EB1, EB3, and CLIP-170, passes newly-formed EEs from the actin cortex to MT-associated dynein via dynactin, where the endosome traffics to the cell interior [126, 127]. EEs undergo a quick drop in pH to between 6.8 and 6.1 upon trafficking to, and fusing with, early sorting endosomes [128]. Here, competing interactions between dynein and kinesin molecules are important for membrane fission of newly-formed recycling and late endosomes (LEs) [129-131]. Vesicle contents are sorted into either Rab4<sup>+</sup> or Rab11<sup>+</sup> recycling endosomes, or sent on to mature into Rab7<sup>+</sup> LEs [132, 133]. The majority of endocytose molecules are sent back to the cell surface through recycling endosomes. Recycling endosomes colocalize with the kinesin-13-family molecule Kif13A, and kinesin traffics the cargo of these vesicles back to the plasma membrane or to the trans-golgi network (TGN) [134].

LEs are sent towards a more destructive pathway, targeting their contents to lysosomes for degradation. These vesicles are bidirectional, suggesting the use both kinesin and dynein molecules to move throughout the cell, although direct interactions with kinesin have not been shown [135]. Dynein binds LEs via dynactin interactions with Rab7-interacting lysosomal protein (RILP) and the oxysterol-binding molecule ORP1L [136]. LEs mature in the span of less than an hour, sustaining various modifications: They increase in size; intraluminal vesicles (ILVs) bud into the LE via ESCRT machinery, sorting ubiquitinated proteins into smaller vesicles for easier degradation; Rab5 is switched out for Rab7, altering its function and recruiting novel effector molecules, such as RILP to bind dynein and LAMP1 to protect the limiting membrane



from degradation; and the vesicle is further acidified to approximately pH 4.8, to assist its fusion with very low-pH lysosomes [137]. At this point, LEs typically continue on to fuse with lysosomes and degrade their contents, although they may also traffic back to the plasma membrane to release their ILVs into the extracellular milieu as exosomes [138]. Lysosomes traffic throughout the cell by way of both dynein and kinesin-1 Kif5B activities [139, 140].

*Intracellular pathogens commonly exploit microtubule functions*

There is considerable pressure on a cell to maintain the functions and activities of MTs and their associated proteins for viability. To this end, many intracellular pathogens have evolved mechanisms to make use of MTs in their own way, either for entry, replication, immune evasion, or combinations thereof. Viruses generally exploit MTs by hijacking motors to reach their preferred site of replication. Both HSV1 and pseudorabies virus (PrV) establish latent infections in cells via transport to the nucleus on MTs by way of their tegument proteins [141-144]. Efficient infection with these viruses requires dynein function [145, 146]. Furthermore, purified HSV-1 capsids directly bind kinesin-1 and kinesin-2 molecules [147], however this interaction requires inner tegument proteins since capsids purified from nuclear extracts do not bind the motors. Vaccinia virus (VV) also uses kinesin-1 to move immature enveloped virions (IEVs) from viral replication factories near the nucleus to the cell surface, where they acquire a second membrane envelope and exit the cell [148]. Research suggests that a VV protein, F12, mimics the structure of kinesin light chain, binding the KHC directly to mediate transport [149, 150]. Alternatively, another VV protein, A36, binds KLC TPRs, suggesting the virus interacts

with fully complexed kinesin molecules for egress [151]. Upon entry into the cytoplasm after envelope fusion with the cell surface, HIV core complexes exploit microtubule networks for cell transport. While the complex requires dynein to move to the MTOC, it is unclear how this occurs [152]. While some have implicated that the HIV capsid protein, p24, is important for MT associations, others have pointed out that capsid availability on the core is unstable and rapidly disassembles as reverse transcription occurs [152, 153].

Bacterial pathogens can also use MT motors to move within the cell as well, although some produce factors to destabilize microtubules entirely. *Shigella* bacteria utilize their type 3 secretion system (T3SS) to introduce the effector molecules into host cell cytoplasm. One effector molecule, VirA, reportedly degrades  $\alpha$ -tubulin monomers, destabilizing MTs and enhancing bacterial entry and movement within the cytoplasm [154-156]. Although it does not have the ability to actively destabilize MTs, *Chlamydia* produces CopN to directly sequester monomeric  $\alpha$ - and  $\beta$ -tubulin molecules, inhibiting the formation of new MTs [157, 158]. Still other bacteria have the ability to completely rearrange the MT network architecture for their own ends. *Listeria*, which is commonly known for its visible comet-shaped actin “tails,” reassigns dynamin-2 to aid in its movement [159]. Dynamin-2 is a GTPase utilized by the cell to properly bundle MT filaments for cell motility [160, 161]. *Salmonella* completely usurps microtubules using its effector molecule SifA. Secreted via T3SS, SifA reforms microtubules entirely into tubules that form around SCVs, called *Salmonella*-induced filament (SIFs) [162].

Effectors SseG and SSeF induce further microtubule bundling, however the specific details of MT restructuring is unclear.

Collectively, these examples showcase a clear dependence of both cells and pathogens on the microtubule network. Using these pathogens as tools to systematically examine the processes required for the regulation and maintenance of MTs will aid our understanding of both the host cell activities requiring MTs as well as the mechanisms of pathogenicity by various infectious agents.

### **Adenoviruses**

Adenoviruses (Ads) are icosahedral, non-enveloped, linear double-stranded DNA-containing viruses belonging to the genus *Mastadenovirus*. Since their original isolation from adenoid tissue in 1954, more than 60 human Ad serotypes have been identified and categorized into 7 subgroups (A-G) based on sequence homology, oncogenicity in rodents, pathogenicity, serum neutralization, and hemagglutination properties [163-169]. Initially identified as a causative agent of acute respiratory disease (ARD), reports indicate that some serotypes cause gastrointestinal, ocular, or urinary tract infections as well [170, 171]. Although some human species cause oncogenicity in rodents, there is no indication that they cause tumorigenesis in human cell lines [172, 173]. Depending on the subgroup, the adenoviral genome varies from 30-40 kbp in size and encodes approximately 40 genes (Table). Adenovirus infection is a useful tool to probe cellular processes, study intrinsic immune responses of the host cell to virus infection, and further understand mechanisms evolved by viruses to evade detection and restriction. This study focuses on a subgroup C virus, Ad5.

*Adenovirus capsid*

The Ad capsid is a 90nm wide, icosahedral capsid made up of 12 structural proteins (Figure 1). The icosahedral shape gives the capsid 2-, 3-, and 5-fold symmetries. The major capsid protein, hexon (protein II), makes up the majority of the viral shell in 720 copies, forming 240 trimers (20 facets, 12 trimers per facet) [174, 175]. Located at each of the twelve vertices of the capsid is the penton complex, made up of penton base (protein III), anchoring trimers of the fiber protein (protein IV). Two more so-called minor capsid proteins, protein VIII and protein IX, stabilize the integrity of the capsid. Proteins VI and IIIa are contained just below the surface of the capsid shell [168], and act as a sort of secondary lattice. Protein VI interacts with the core protein V, which is associated with the viral dsDNA genome, and is delivered to the nucleus along with the DNA. Five other proteins are found on the interior of the capsid along with the genome: protein VII, Mu ( $\mu$ ) protein, protein IVa2, the terminal protein (TP), and the adenoviral protease.

The capsid of mature virions can be pictured as having three layers, so to speak: an outer layer, an inner layer, and the genome-containing interior [176]. The interior of the virion contains the genome and the core proteins pV, pVII,  $\mu$ , pIVa2, a viral protease and TP [177-181]. This region is highly disordered, and thus not crystallized, suggesting the genome is not as tightly packed as other viral genomes such as herpes viruses or bacteriophage. The C-terminus of pV, however, is ordered at the inner layer of the capsid, found in a ternary complex with pVIII and pVI at the vertex region [176]. The current model depicts pV as a bridge between the DNA core and the capsid shell, where the

basic, disordered N-terminus of pV interacts with the genome, while its C-terminus interacts with pVI and pVIII in the inner layer. Both  $\mu$  and pVII are highly associated with the viral genome, and believed to be critical for proper DNA condensation and packaging into the capsid [182]. Although it interacts with the viral genome to efficiently encapsidate the DNA into developing capsids, pIVa2 is less abundant in copy number within the mature virion. Reports indicate that pIVa2 is a critical initiator of the viral major late promoter (MLP), regulating late viral gene expression and driving the expression of the structural capsid proteins [183]. TP facilitates viral DNA circularization during replication for efficient packaging into the capsid, and covalently attaches to the 5' ends of the viral genome. During viral entry, TP is important for priming DNA replication once the virion has delivered its genome to the nucleus [184].

The adenoviral L3-23K protease (AVP) is important during viral replication, cleaving multiple viral precursor polypeptides into their active forms [185, 186]. AVP requires both viral DNA and a C-terminal fragment of pVI as cofactors for its proteolytic activity. The protease, when bound to viral DNA, interacts with the precursor form of pVI. It cleaves both the N- and C-termini of pVI, converting pVI into its active form. The C-terminal fragment of pVI (pVIc) is then utilized by AVP as a cofactor to move along viral DNA to encounter other viral precursor proteins, pVII, pVIII, TP and  $\mu$  [187, 188]. Protease activity is essential to produce functional mature virions [189]. A viral mutant containing a temperature-sensitive form of AVP (P137L mutant, called Ad2-ts1) does not fold properly when replicated at the non-permissive temperature (39°C), and is unable to properly process viral precursor proteins [189, 190]. Virions produced at the non-

permissive temperature are taken up by cells, but do not penetrate into the cytoplasm, suggesting that virion maturation enacted by the protease is essential for viral entry. Exposing virions to reducing and alkylating conditions prior to infection, which inactivates purified protease activity *in vitro*, reduces infectivity by nearly 5-fold, as assessed by plaque assay [191]. These data indicate a possible role for the protease during entry, after maturation. However, the effects of this treatment on other structural proteins is unknown, and as such the effects of this treatment cannot be directly attributed to protease activity. Additionally, other have shown that AVP contains both deubiquitinating and deISGylating activity *in vitro*, and global deubiquitination of cellular proteins occurs 12 hours post-infection [192]. Similar examinations at 6hpi showed no deubiquitinating activity compared to mock-infected cells, however deubiquitination was not examined at times corresponding to initial virion entry. Whether protease activity is important during entry, either through further protein processing or via deubiquitinating function, still remains an area worthy of investigation.

Twenty triangular facets make up the outer layer of the capsid, with each facet containing a three-fold axis of symmetry. The majority of the outer layer of the shell is composed of hexon, with each capsid facet composed of 12 hexon trimers (referred to hereafter as just hexon) [193]. Hexon is organized into groups of four structurally unique capsomers, referred to as an asymmetric unit (AU) [194]. Each hexon within the AU is assigned a number, 1-4, depending on its position relative to the AU. Hexon in position 1 is made up exclusively of peripentonal molecules, and hexon within the other positions make up the remainder of the face, containing nine hexon (referred to as the group of

nine, or GON) [195]. Hexon has trimeric symmetry on the outer surface, and the base of the molecule forms a pseudo-hexagon, with each edge alternatingly made up of one or two monomers. The formation and layout of the AUs produces eight distinct hexon-hexon interactions, suggesting an inherently complex assembly pattern.

Penton base and fiber proteins (collectively referred to as penton) are present in the outer layer of the capsid at the vertex of each facet, making up the five-fold axes of symmetries [196, 197]. Penton base acts as the base of the vertex, interacting with the five peripentonal hexons. Fiber is a long, flexible shaft protruding from the center of the penton base. The fiber shaft is a homotrimer making up a unique  $\beta$ -spiral, and extends approximately 300Å from the capsid, culminating in a knob at the end of the protrusion [198]. Both fiber and the penton base are critical for adenovirus attachment to cell receptors.

Proteins pIIIa and pIX are also on the exterior of the capsid, and thought of as cement proteins to stabilize the capsid shell [199, 200]. pIIIa strengthens hexon-hexon interactions between the three hexon AUs making up each facet. There are five monomers of pIIIa arranged symmetrically under each penton base vertex, giving 60 copies of pIIIa per virion. The protein is hypothesized to stabilize the capsid vertexes and the genome once the viral DNA is packaged into the assembled virion, as well as maintain the integrity of the hexon facets. Six pIIIa molecules surround each facet border along the two-fold axes of symmetry, with two proteins (one from each facet) stabilizing the interaction between a pair of facets. The protein stretches from between two peripentonal hexons along the outer edge of a capsid face, ending in an antiparallel, four-

helix bundle between GON-GON interfaces. pIIIa is predicted to act as a tape measure protein, similar in function to the P30 protein of the bacteriophage PRD1 [201], and interactions between pIIIa N-termini and the L1 52-55K scaffolding protein facilitate genome packaging [202].

A second stabilizing protein, pIX, is a mainly extended molecule, stabilizing the interactions between hexons [203-205]. Four pIX molecules within a facet make up four triskelion structures: three start at the peripentonal hexon and stabilize hexons within an AU in a local, quasi-threefold symmetry, and one stabilizes the three AUs at the center of the facet in a strict three-fold symmetry. In total, the capsid contains 240 pIX molecules making up 80 triskelions, 60 of which make up the first type, and the other twenty making up the second type. Together, pIIIa and pIX make up an almost entirely contiguous framework surrounding the hexons within a GON [176].

pVI, pVIII, and pV make up a secondary lattice just underneath the outer layer [176]. This inner layer, while not as contiguous as the proteins making up the outer layer, stabilizes the hexon outer shell, including peripentonal hexons. pVIII is found in the inner layer as two structurally different conformations. The first forms a complex with pVI and pV at the capsid vertices, associating with the peripentonal hexons. The second interacts with pVI molecules found at hexon-2 molecules of the AU. Both conformations associate with the border of hexon GONs, mediating the interactions of peripentonal hexons with GON hexons. pVIII has also been hypothesized to act as a tape measure protein, since it is within the capsid interior, similar to p30 of PRD1 [201]. The final protein within the



inner shell is pV, which interacts with pVI and pVIII in the inner shell as well as viral DNA within the center of the virion.

Previous reports implicate many varied roles for pVI, including membrane rupture, endosomal escape, nuclear transport, and the use of a C-terminal fragment as a cofactor for adenoviral protease activity in cleaving viral pre-proteins to produce mature virions [187, 188, 206-211]. The crystal structure of the pVI N-terminus was recently solved in the context of a virus, indicating one copy of pVI tightly associated with the base of each peripentonal hexon [176]. Five pVI molecules act as “molecular glue” between each peripentonal hexon surrounding a penton base, binding one peripentonal hexon to the peripentonal hexon from an adjacent AU, and joining them to the hexon-4 molecule on the adjacent AU. Crystallized pVI is also found associated with hexon-2 subunits of the AU. While the crystal structures account for approximately 120 of the reported ~350 copies of pVI within a mature virion, other pVI molecules may associate with the viral DNA that are highly disordered, and thus would not be seen in the crystal structure. Interestingly, while previous papers report that the N-terminus of mature pVI adopts an amphipathic helix important for its membrane rupture activity, this configuration is not present in the crystal structure of pVI within the virion. The inability to form a critical helical domain while inside the mature capsid may be due to conformational constraints within the small volume of the virion, and the amphipathic helix may form once pVI releases from the interior of the capsid shell and/or associates with endosomal membranes.

*Adenovirus vectors as vaccines*

Adenoviruses infections are generally mild and self-limiting in healthy populations, but can be devastating to the elderly and immunocompromised individuals, such as those with AIDS or patients undergoing chemotherapy [212-216]. Since these infections are generally self-limiting, replication-defective Ads as vaccine vectors is generally regarded as safe, and as such are being thoroughly investigated [217-221]. The Ad genome is well-characterized, and can be manipulated relatively easily compared to other recombinant vectors. Ad vectors have a broad tropism, viral genomes do not integrate into host genomes, and elicit a potent inflammatory response. These replication-defective vectors are deleted for E1, an essential gene for viral replication. E1-deleted Ad vectors are cultured in complementing HEK-293 cells, which are a transformed cell line containing the E1 gene. Our Ad vectors are also deleted for E3, allowing for the insertion of foreign DNA up to 8kb without affecting the production of viral progeny.

The proinflammatory responses stimulated by Ad vector transduction produces robust adaptive immune responses in the host, including extremely potent CD8<sup>+</sup> T cell activation and high antibody production [219, 222]. Intracellular recognition of recombinant Ad vectors by TLR activation, virus-induced damage, and cytosolic DNA sensors induce the production type I interferons and stimulate the secretion of a number of pro-inflammatory cytokines, including IL-1 $\beta$ , IL-6, IL-12, TNF $\alpha$ , and IFN- $\gamma$  [223]. These vectors by themselves induce both cellular and humoral responses, eliminating the necessity for additional adjuvants during vaccination. Cytokine production is independent of genome replication, as UV-inactivated vectors still elicit the production of these

molecules [224, 225]. In addition to producing a robust pro-inflammatory response, recombinant Ad vectors containing a transgene persistently express the protein, and is detectable for up to a year post administration *in vivo* [226]. Low level expression of the transgene maintains functional CD8<sup>+</sup> effector T cell populations in the immunized animal, which is commonly found in persistent low level antigen expression models, such as persistent CMV infections. Studies also suggest that CD8<sup>+</sup> effector memory T cell populations are strong correlates of disease protection.

Ad vaccine vectors have been explored for a variety of pathogens currently of high interest, including HIV, Ebola, and influenza [227-229]. Current-generation Ad vaccine vectors exhibit high immunogenicity, but lack the efficacy to mount an optimal response. Many of these vectors are based on one of the most characterized serotypes of adenovirus, human Ad type 5 (Ad5), but more than half of the population have neutralizing antibodies against Ad5, limiting the capacity of these vectors to effectively transduce host cells [230]. To circumvent this, ongoing work is exploring the generation of chimeric Ad vectors that are primarily Ad5 but contain capsid epitopes from other Ad serotypes that are less prevalent in human populations [231]. Utilizing other so-called rare human serotypes that are less seroprevalent, as well as Ad species originating from chimpanzees, are also under investigation [232]. Another strategy under current development is introducing antigenic sequences into the exposed loops of hexon molecules called hypervariable regions (HVRs), displaying the peptide sequence directly on the capsid surface. Neutralizing antibodies are heavily skewed towards recognizing any of the seven HVR sequences, thus replacing one or more of these HVRs with

antigens from a vaccine's intended target could improve antibody development against the organism. By employing a combination of these differing strategies, as well as utilizing the immunogenicity of the Ad capsid to present antigens of the pathogen of interest directly on the capsid surface, we can skew future generation vectors to provide a more beneficial response against the targeted pathogen.

The effects of Ads on human health – both by infection and as a vaccine – are substantial, however little is known about its interactions with host cells to productively establish an infection. Virus cell entry represents one of the first opportunities for the host to respond to infection. The processes of pathogen detection and restriction by the host, as well as strategies utilized by adenovirus to evade these constraints within its target, are critical events to understand to generate better therapies.

#### *Adenovirus cell entry*

##### *Adenovirus receptors*

In order for adenoviruses to begin infecting a target cell, the virion must be endocytosed. To do this, two interactions must occur between the viral capsid and host cell receptors. The first interaction occurs via a high-affinity interaction between the knob domain of the Ad fiber protein and a subgroup-dependent primary attachment receptor on the host cell [233, 234]. Ad subgroups A, C, D, E, and F primarily use the Coxsackievirus B and Adenovirus Receptor (CAR) to mediate cell attachment [235, 236]. In addition to CAR, subgroups C and D can utilize other cell surface receptors as well, depending on the cell type. Subgroup C viruses have been suggested to use heparin sulfate proteoglycans (HSPG), MHC class I, and VCAM-I as interacting ligands via a basic

KKTK motif present within their fiber proteins, and subgroup D can use sialic acid and/or CD46 [236-243]. Subgroup B viruses do not appear to use CAR, and instead interact with CD46, HSPG, CD80/86, and Dendrolysin 2 for their initial binding [244-246].

More recently, studies suggest that viral capsids associating with soluble, extracellular cofactors aid in primary attachment during systemic infections [247-250]. Specifically, the failed use of subgroup C virus Ad5 in a gene therapy study revealed that this virus has a CAR-independent tropism for liver cells, instead utilizing various blood coagulation factors including Factors VII, IX, and X, as well as the complement component C4-binding protein to mediate transduction into hepatic cells [249].

Once the Ad fiber has successfully mediated attachment to cells through primary receptor interactions, a second, lower-affinity interaction between an Arginine-Glycine-Aspartate (RGD) motif found within the Ad penton base and  $\alpha_v\beta_3$  or  $\alpha_v\beta_5$  integrins induces cell internalization of the virus. Integrin binding induces a cell signaling cascade through phosphoinositide-3 kinase and Rho GTPase activation, initiating virion uptake through clathrin-mediated endocytosis [251-255]. Observations indicate that the interactions between these viral proteins and different receptors mediate virion trafficking in the cell to certain compartments [248, 256]. Subgroup C viruses, including Ad5, only traffic through early endosomal compartments before penetrating into the cytosol [256], where subgroup B viruses must traffic to late endosomes/lysosomes before escaping from these vesicles [248, 257]. Studies also suggest that Ad2 (subgroup C) interactions with  $\alpha_v$  integrins mediate macropinocytosis in a clathrin-independent

process, alongside clathrin-mediated endocytosis [258]. Other reports show that this may be an important mechanism of entry for certain adenovirus species, as inhibitors of macropinocytosis prevent the entry of the subgroup B virus Ad3 into cells. Furthermore, Ad3 interactions with  $\alpha_v$  integrins, along with CD46, stimulates plasma membrane ruffling of the host cell, facilitating increased fluid uptake [259].

#### *Adenovirus uncoating and endocytosis*

Once Ad penton engages integrins and endocytosis has begun, the viral capsid must partially dissociate for infection to proceed. Fiber-bound CAR receptors have fluid movements within lipid membranes, but penton base-integrin interactions are more rigid and spatially confined [260]. These movements induce mechanical stress to the viral capsid: Ad fiber is pulled by the drifts in CAR and shed at the cell membrane, independent of the initiation of endocytosis [260, 261].

Ad internalization initiates further disassembly of the capsid in a stepwise manner [262]. Although initial studies indicated that  $H^+$  influx into endosomes to acidify the vesicle compartment initiates capsid uncoating for certain serotypes (especially those that traffic to late endosomes) [262-265], more recent observations indicate that many serotypes, including Ad5 and other subgroup C Ads, do not depend on acidification [256]. Ad5 escape from endosomes occurs within 15 minutes of internalization, signifying escape from early endosomal compartments, before the virus would encounter low pH [191, 206, 256, 262, 266]. Furthermore, adding weak bases to viral infections, such as ammonium chloride, does not affect capsid disassembly of subgroup C viruses [267].

*Adenovirus membrane rupture*

After Ad is internalized by endosomes and begins the process of uncoating, the virus must penetrate into the cytosol to continue the infection process. The virus needs to lyse endosomal membranes to achieve this. Initial studies linked the role of viral uncoating and endosomal rupture using a temperature-sensitive mutant of the subgroup C virus Ad 2 (ts1-39), which cannot uncoat when passaged at the non-permissive temperature (39°C), due to hyper-stabilization of the capsid [189, 268]. This virus contains a point mutation in the L3-23K viral protease (P137L) that fails to properly fold at the non-permissive temperature, and is not properly packaged into nascent virions [189, 190, 269, 270]. The protease functions within completely assembled virions to cleave six Ad capsid pre-proteins into their mature forms [271-273]. Immature, uncleaved virions have increased capsid stability, causing a defect in viral uncoating, membrane rupture, and endosomal escape [272]. Ts1-39 virus is either recycled through the endocytic network back to the cell surface, or traffics to late endosomes/lysosomes and is degraded [191, 256].

More recent studies have linked capsid disassembly to endosomal lysis. Dismantling of the viral capsid releases the interior capsid protein VI (pVI), which has been recently shown to possess all necessary membrane lytic activity for viral rupture of the endosome [206, 208, 274]. Release of pVI from the capsid interior is dependent on the uncoating process, and pVI can be detected at the cell surface within 5 minutes of virus internalization [209]. Antibodies against pVI do not bind intact virions, thus detection of pVI at the cell surface further supports that low pH is dispensable for capsid

uncoating [209, 260], even indicating that pVI activity occurs prior to the . Infections using an Ad5 mutant that can bind integrins without binding CAR (Ad5-RGD4C) links pVI exposure to the capsid-receptor interactions at the cell surface. Ad5-RGD4C-infected L929 cells, which do not express CAR, revealed less pVI exposure compared to L929 cells overexpressing CAR, though both cell types endocytosed similar numbers of virions [260].

Protein VI is a structural protein, with approximately 342 copies present in the capsid [175, 275], and is implicated in a number of functions during the Ad life cycle. Mature, cleaved pVI is suggested to localize underneath each hexon trimer within the mature virion [174, 204], and is necessary and sufficient to disrupt membranes *in vitro*, as antibody neutralization targeting pVI prevents Ad membrane rupture [206, 208]. Unlike other viruses, such as reovirus, which form small pores in endosomal membranes [276, 277], pVI wholly fragments endosomes, as evidenced by translocation of high molecular weight molecules, including 70 kDa dextrans or whole parvoviruses [265, 278]. The membrane rupture activity is contained within a small stretch of amino acids at the N-terminus of the protein, corresponding to residues 36-53 [206, 208], which is predicted to be part of an amphipathic alpha helix. This stretch of residues increases the affinity of pVI to associate with membranes and induce positive curvature on the membrane, thus causing the membrane to fragment. The amphipathic alpha helix, along with 3 other alpha helices in the pVI N-terminus, embeds into the membrane in a parallel manner, inducing positive membrane curvature stress to fragment lipid vesicles [207, 208]. Although recent structure data suggest that pVI structure does not contain an amphipathic



alpha helix while associated with the viral capsid [176], this does not preclude a conformational change occurring once pVI has been released from the partially dissociated capsid. Deletion of this stretch of residues or mutation of conserved tryptophan residues limits membrane interactions and reduces fragmentation of liposomes *in vitro*, although a mutant pVI lacking the amphipathic alpha helix still retains some lytic activity [206-208].

In addition to its importance in membrane rupture, data also implicate that pVI is critical for efficient endosomal escape. A mutant adenovirus that has a mutated PPxY motif in the C-terminal portion of the protein (called Ad5-M1) does not hamper the ability of the virus to fragment endosomes, but restricts Ad infectivity 20-fold. This reduction in infectivity corresponds to an increased colocalization of Ad5-M1 with ruptured endosomes over the course of infection (Figure 2). How the PPxY motif facilitates virion penetration into the cytosol is still under investigation.

#### *Galectins as markers for vesicle lysis*

In intracellular bacterial infections, endosomal lysis is evidenced by the accumulation of proteins containing sugar-binding moieties, called galectins [279]. Galectins are primarily cytosolic proteins, found within the cell in a diffuse manner. N-linked glycans present at the cell surface, and thus in the luminal side of an endosome, are normally inaccessible to the sugar-binding galectins. However, upon vesicle fragmentation, galectins aggregate at the sites of injury to bind the N-linked glycans, and these can be visualized as discrete puncta using immunofluorescence. During *Salmonella* infection, galectins -3, -8, and -9 are recruited to sites of endosomal rupture by the

bacterium. Gal8 knock down by siRNA enhances *Salmonella* replication [72]. The investigators found that knocking down gal8 expression inhibits ubiquitin binding proteins NDP52 and p62 from binding ubiquitinated bacteria, thus preventing autophagosome formation around the pathogen. The importance of gal3 and gal9 recruitment to ruptured endosomes was not elucidated, but siRNA knock down of either form had no effect on bacterial replication.

#### *Autophagy activation*

Very little has been assessed regarding the role of autophagy during adenovirus infection, and of those results, its function has only been looked at at very late times post-infection (after 24 hours), by which time virus trafficking towards the nucleus and genome delivery has already occurred [280-283]. The role of autophagy during adenovirus entry remains an open field. Data from our lab suggests that the 20-fold infectivity defect of Ad5-M1 occurs as a result of an inability to quickly escape endosomes, and is subsequently sequestered by autophagosomes and targeted to lysosomes for degradation (Figure 3). *In vitro* ubiquitination experiments suggest that pVI-WT is oligo-ubiquitinated with 2x or 3x ubiquitin appendages after release from the capsid. However, the same studies performed with pVI-M1 suggest the mutant protein is not as extensively ubiquitinated [209]. Although this makes sense as PPxY motifs are frequently ubiquitinated in natural cellular processes [284, 285], it suggests that pVI ubiquitination is not the key mechanism of capsid targeting to autophagosomes, as is seen with appending ubiquitin to bacterial pathogens. However, other data from our lab suggest that it is the host proteins recruited to endosomes upon membrane damage that

signal autophagy formation. Similar to what is seen during *Salmonella* infection, we find that galectin-8 knock down by siRNA restores mutant Ad5-M1 infectivity to Ad5-WT levels. However, whether the other mechanisms of autophagy recruitment seen in bacterial infections are also important during adenovirus infection remain unknown.

#### *Microtubules facilitate viral transport upon entry*

Nucleus-bound viruses must translocate across relatively large distances through the cytosol to get from the cell surface to the nucleus. The cytoplasm is extremely dense, and free movement or diffusion through the medium is limited by a number of organelles, cytoskeletal components, and other proteins. Studies using fluorescence recovery after photobleaching (FRAP) suggest that molecules greater than 20nm in size are essentially immobile in the cytoplasm [286, 287]. Adenovirus particles are 90nm in diameter, suggesting that movement through the cell cytoplasm by diffusion is not an effective way to deliver its viral DNA to the nucleus. To effectively move through the cell, Ads exploit the microtubule network already established within the cell. Chemical depolymerization of the microtubule network using nocodazole severely restricts Ad infectivity.

Furthermore, inhibiting the activity of either dynein or kinesin motors through siRNA knockdown or antibody injection limits Ad infectivity, suggesting that both motor proteins are intimately involved in entry [288, 289]. Although some literature indicates the interaction of viral capsid proteins with motor proteins is important for efficient entry, the specific contributions of microtubule motors has not been explicitly uncovered.

Hexon that is exposed to acidified environments reportedly interacts with dynein in *in vitro* experiments [290]. Hexon, fiber, pIIIa, and pIX all bind kinesin in pull down assays

[291]. Furthermore, pVI interacts with microtubules in a PPxY-dependent manner, however whether this is due to interacting with dynein, kinesin, or some other interaction is yet to be revealed [209]. Current data suggest that dynein traffics the virus towards the microtubule organizing center (MTOC) near the nucleus, and kinesin appears to play a role in nuclear import, once the virus accumulates at the nucleus.

#### *Adenoviral genome delivery into the nucleus*

Once the partially uncoated virus has ruptured endosomes and penetrated into the cytosol, it must traffic to the nucleus and deliver its genome. Literature indicates that virus movement along microtubules is critical for accumulation at the nucleus. The nuclear import factor chromosomal region maintenance 1 (CRM1) competes with Ad for microtubule interactions, enhancing capsid dissociation at the nucleus to facilitate genome import [292]. Impeding CRM1 function increases Ad accumulation at the MTOC and spindle poles, and restricts genome delivery by preventing disassembly of the capsid. Once free virus is transported to the nucleus, the capsid docks at the nuclear pore complex (NPC), presumably through interactions of hexon and the NPC protein Nup214 [293]. Once docked, kinesin interacts with pIX present on the capsid and Nup358 at the NPC. Opposing forces between the Nup358-kinesin-pIX and hexon-Nup214 interactions disrupts the NPC and pulls apart the viral capsid, freeing the viral genome from the capsid core [291]. Soluble histone H1 proteins associate with conserved acidic residues on hexon, and subsequently other nuclear import factors bind and import the viral DNA into the nucleus.

*Virus replication and nascent virion production*

Once the genome is in the nucleus, DNA transcription and the replication of new virions occurs. The pVII-associated viral DNA complex interacts with nuclear factors, including TBP-associated factor 1 (TAF-1), remodeling the viral chromatin structure to facilitate transcription of initial viral replicating genes [294, 295]. Altering the viral DNA is essential for replication, as early gene expression is severely inhibited in cells knocked down for TAF-1. RNA polymerase II initiates genome transcription, commencing the first stage of viral replication. Within hours, replication of host cell DNA ceases, focusing only on viral replication.

Viral transcription is temporally divided into two phases – early and late – with viral DNA replication acting as the dividing event. Early transcripts are responsible for changing the host cell environment to optimally generate new virions, restricting intrinsic immune responses generated by the host, and enhancing the transcription of late genes necessary for Ad replication and capsid assembly. Early transcription is further broken into three subsets: immediate-early, early, and intermediate. In the immediate-early stage, RNA polymerase II transcribes the viral E1 gene into E1A mRNA. E1A transcripts are exported into the cytoplasm via CRM1-dependent activity [296]. Transcript translation and subsequent import of E1A product into the nucleus drives the transcription of the other early genes E1B, E2, E3, and E4 via the activation of host cell transcription factors [297]. E1A also forces the cell to progress into the S phase of the cell cycle, affecting host cell gene expression and promoting the transcription of E2 genes, one of the genes transcribed during the early stage of transcription. In addition to E1A activity, E1B

translation generates two polypeptides, E1B-19K and E1B-55K. The former inhibits infected cell death by blocking the apoptosis inducer Bax [298]. The latter protein forms an E3-ubiquitin ligase complex with E4ORF6 to target the tumor suppressor p53 and components of the MRN complex, a DNA damage repair system, for proteasomal degradation [299]. The E1B/E4 complex also controls selective export of late viral mRNAs into the cytoplasm for translation [300].

After E1A activates their transcription, mRNA for the early genes E2, E3, and E4 undergo alternative splicing processes, are translated, and their gene product begin to enact their functions. The E2 gene encodes for three molecular components absolutely essential for replication: DNA binding protein (DBP), the precursor terminal protein (pTP), and viral DNA polymerase (Ad Pol). These proteins, along with host transcription factors NFI and Oct-1, enhance the initiation of viral transcription over 100-fold [301]. E3 gene products restrict the cell from producing antiviral defenses by retaining MHC class I molecules in ER and down-regulating cell surface expression of TNF-related apoptosis-inducing ligand (TRAIL), Fas receptor, and epidermal growth factor receptor (EGFR). Activation of the E4 gene produces a number of polypeptides from different open reading frames (ORFs). E4ORF6 complexes with E1B-55K as described above. E4ORF3 targets components of the MRN complex to aggresomes and disrupts promyelocytic leukemia (PML) bodies in the nucleus, inhibiting these structures from sequestering and restricting viral replication machinery [302, 303]. Deleting E4ORF3 severely limits adenovirus replication. E4ORF1 binds and activates MYC, enhancing the

production of glycolytic enzymes and increasing the biosynthesis of nucleotides [304]. E4ORF4 negatively regulates E1A-activated transcription [300].

Once the functions of the early genes predispose the cell for optimal virion production, DNA synthesis can commence. The intermediate genes IVa2 and IX are transcribed and translated [183, 305, 306]. These viral genes are the only two genes that do not produce multiple splice variants, translating only one gene product. The gene product of IVa2 is the first virally-expressed protein to contain DNA-binding activity, and is restricted to the nucleus after translation. Protein IVa2 dimerizes and binds to sequences upstream of the major late promoter (MLP), acting as transcription factor to activate the MLP and transcribe late genes L1-L5, which encode capsid structural proteins [307]. The function of pIVa2 likely enhances the binding of other proteins to viral DNA. *In vitro* assays indicate it has no transcriptional activity by itself, and it requires the assistance of host regulatory factors to stimulate MLP promoter activity. In addition to its transcriptional activity, pIVa2 aids in viral DNA packaging into maturing capsids [180].

Although the N-terminus of intermediate gene IX product pIX is important for capsid incorporation, its C-terminal alanine-rich region and leucine zipper domain acts as a trans-activator of both viral and host genes [305, 306]. It is the only viral protein to act as both a structural protein and a transcription factor. Although it contains no DNA-binding activity of its own, it enhances transcription at both viral and cellular promoters containing TATA sequences, including the MLP. Vectors deleted for pIX produce less fiber capsid protein during the course of infection and cannot incorporate full-length

genome into assembling virions compared to pIX-sufficient controls, indicating its importance during virion production [308]. Deleted vectors also exhibit a two- to three-fold decrease in virus production after expansion in complementing cells. Its activity is enacted even when expressed in uninfected cells by plasmid transfection, indicating that its function occurs in the absence of other viral proteins. In addition to its transcriptional activities, the pIX leucine repeat is a critical mediator of PML-body sequestration, keeping them from accumulating at sites of viral replication [309]. It may act sequentially with E4orf3 to evade PML-stimulated restriction of DNA replication. Levels of pIX expression change during the course of replication, starting at low levels at the intermediate stage and increasing to higher levels after DNA replication has begun [310]. This change in expression levels is inversely proportional with the levels of E1B transcription, as the E1B coding sequence reads through both gene IX's promoter and coding sequence [311]. Once E1B transcription subsides after DNA replication begins, pIX expression dramatically increases.

Simultaneously with intermediate gene expression, Ad Pol, pTP, and DBP act in concert to replicate viral DNA, producing up to  $10^6$  nascent genomes within 40 hours, for both increased transcription and packaging into assembling capsid molecules. The TP-bound 5' ends of the DNA encounter a pTP-Ad Pol complex containing a covalently-bound cytosine molecule to the pTP [312]. Two host transcription factors, nuclear factor I (NFI) and nuclear factor III (Oct-1) each independently enhance replication initiation, with an additive effect of greater than 100-fold [313]. Late gene mRNA transcripts produce the structural capsid proteins necessary for virion assembly, which occurs within



the nucleus, where the viral genomes reside. Fiber trimers and penton pentamers mediate their own import into the nucleus via encoded nuclear localization signals (NLS). Hexon proteins have no NLS in their sequences, and upon trimerization rely on import into the nucleus via pre-protein VI [211]. Once all of the capsid proteins are present within the nucleus, assembly is initiated. Empty capsids containing all of the outer shell pre-proteins and viral protease molecules assemble without DNA present within the capsid, then pIVa2 and late gene-encoded scaffolding proteins mediate viral genome insertion into capsids. Conflicting evidence muddies the complete understanding of capsid assembly. Studies inhibiting DNA import into capsids results in unassembled capsid molecules within the nucleus, suggesting capsid assembly is not spontaneous and that there is a discrete link between DNA interactions with capsid proteins and capsid assembly [314]. Regardless of how these molecules assemble and import viral DNA, once DNA is present within the capsid, the viral protease cleaves all pre-proteins into their final forms, converting the virion into a mature state, ready for infection. In replication-competent adenoviruses, the adenoviral death protein (ADP) induces cell apoptosis, releasing the newly-created virions into the extracellular milieu [315].

## CHAPTER II

### MATERIALS AND EXPERIMENTAL METHODS

**Cell Lines and Viruses.** Tissue culture reagents were obtained from Mediatech and HyClone. HeLa and A549 cells were purchased from ATCC. 293 $\beta$ 5 cells were a kind gift from Dr. Glen Nemerow [316]. MEFs were a kind gift from Dr. Ed Campbell. HeLa cells stably over-expressing KLC1 TPR or HA-tagged pIX sequences were created by transfecting 293 $\beta$ 5 cells with retroviral EXN vectors (Cherry-C1AJ or EXN vectors, respectively) containing the sequence of interest along with pCig and HEF-VSVg packaging plasmids. Retrovirus produced and isolated from these cells were then used to transduce HeLa cells by spinoculation and incubation for 5 hours. Cells were rested for 48 hours, then selected for transgene integration with G418 sulfate solution (MP Biochemicals). Stable HeLa cells were maintained in G418 and assessed for stable integration by immunoblot. HeLa, 293 $\beta$ 5, A549, and stable HeLa cells were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, 1 mg/ml streptomycin, 0.25  $\mu$ g/ml amphotericin B, non-essential amino acids, 1 mM sodium pyruvate, 10 mM HEPES buffer and 2 mM glutamine. The Ad5-WTgfp and Ad5-M1gfp viruses were previously described [209]. Ad5-WTluc was generated by recombination using Ad5-WT in a BAC and the pShuttle-LucCP vector. Ad5- $\Delta$ pIXgfp virus was generated by recombination using Ad5-WT backbone in a BAC and the pShuttle-CMV eGFP vector digested with MfeI and re-

ligated to remove the pIX coding sequence. All viruses were propagated in 293 $\beta$ 5 cells and purified from cellular lysates by double banding in cesium chloride gradients and dialyzed in 40 mM Tris, 150 mM NaCl, 10% glycerol, and 1 mM MgCl<sub>2</sub> (pH 8.2) [206]. Viral concentrations were determined by Bradford assay (Bio-Rad Laboratories, Inc.) and aliquots were flash frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ .

**Reagents, siRNAs, and Antibodies.** Bx795 was purchased from InvivoGen. Nocodazole (NOC) and DMSO were purchased from Sigma-Aldrich. Dorsomorphin and STO-609 were purchased from Cayman Chemicals. SB202190 and JNK inhibitor I-II were obtained from Calbiochem. Ciliobrevin D was purchased from EMD Millipore. NOD1 and NOD2 siRNAs were obtained from Dharmacon. Control siRNA was purchased from Cell Signaling. DLC1 and BAG3 siRNAs were purchased from Santa Cruz Biotechnology. KLC1 siRNA was kindly provided by Ed Campbell. The following primary antibodies were used: rabbit LC3, mouse  $\beta$ -actin (Sigma-Aldrich), mouse galectin-3 (BD Biotechnology), and rabbit CD107a (LAMP-1, Thermo Scientific). Biotin substrate

**Infectivity Assays:** Cells were plated in 24 well plates to yield  $0.5\text{--}1 \times 10^5$  cells/well the day of infection. Cells were collected 18-24 hours after infection, pelleted, and resuspended in buffer (PBS with 1% FBS + 0.1% sodium azide) and GFP<sup>+</sup> cells were counted via flow cytometry (Accuri or Canto).

For drug-treated cells, cells were pretreated for with the specified concentrations of drug for 30 minutes: Bx795, 2.5, 5, 7.5, or 10  $\mu\text{M}$ ; Nocodazole, 30 $\mu\text{M}$ ; Ciliobrevin D, 50 $\mu\text{M}$ ; Dorsomorphin, 40  $\mu\text{M}$ ; STO-609, 1  $\mu\text{g/ml}$ ; JNK I-II, 10  $\mu\text{M}$ ; and SB202190, 20

$\mu\text{M}$ . After pretreating, cells were infected in the presence of drug for one hour, then washed and treated with drug for two more hours, at the same concentrations. Cells were washed three times with PBS and fed media without drug for overnight incubation.

For Ad12 pre-existing immunity assays, cells were transduced with Ad5gfp (20 ppc) or Ad12 (50,000 ppc) overnight in the presence of cord blood diluted in DMEM to 1:16 the original concentration. The next day, cells were washed, fixed with 10% paraformaldehyde in 0.159M PIPES (Sigma) solution for 10 minutes, blocked and permeablized with 10% FBS and 0.5% saponin (Sigma) PBS solution for one hour, and stained to detect infected cells. Ad12-infected cells were stained with rabbit Ad12 antisera and 488-conjugated goat anti-rabbit secondary antibody (Life Technologies). Cells transduced with Ad5gfp were mock-stained. All cells were incubated with DAPI to label cell nuclei. Fluorescence was assessed and imaged by standard fluorescent microscopy, and the percentage of infected cells were quantified by hand using ImageJ.

**siRNA Knockdown:** HeLa cells were plated in 24 well plates and transfected with each siRNA using Lipofectamine 2000 (Invitrogen). Cells were transfected with 20 pmol (Day 1) and with 30 pmol (Day 2) with each siRNA per well. Cells were infected seventy-two hours after the first transfection.

**Immunofluorescence Microscopy:** A total of  $3.5 \times 10^4$  cells were plated on glass coverslips. The next day, cells were chilled on ice for 30 minutes and infected with fluorescently-labeled  $3 \times 10^4$  vp/cell on ice for 1 hour. After virus binding, cells were washed three times with ice-cold PBS, then shifted to  $37^\circ\text{C}$  with pre-warmed medium to allow for virus internalization. At different times post-virus internalization, the cells were

washed with PBS and fixed with 4% paraformaldehyde (Electron Microscopy Sciences) in 0.159M PIPES buffer (Sigma) for 10 min. After all time points were fixed, cells were blocked and permeabilized for 1 hr in PBS with 10% FBS and 0.5% saponin (Sigma). Staining with specific mono- or polyclonal antibodies was performed in 10% FBS with 0.5% saponin for 1 hr. Secondary Alexa Fluor 568-conjugated (Invitrogen) and DyLight 649-conjugated (Jackson) antibodies were used. Viruses were pre-labeled with DyLight 488 NHS-Ester Fluorophores (Thermo Scientific), according to the manufacturer's protocol prior to use. DAPI was used to counterstain nuclei before coverslips were mounted on glass slides with Fluoro-Gel (Electron Microscopy Sciences). Z-stack images were acquired using identical parameters for each time point with a DeltaVision microscope (Applied Precision) using a CoolSnap HQ digital camera (Photometrics) with a 1.4-numerical aperture (NA) 100× objective lens, and deconvolved with SoftWorx deconvolution software (Applied Precision). Images were assembled using IMARIS and ImageJ softwares. Virus particles, LC3 puncta and galectin puncta three-dimensional surfaces were acquired using IMARIS software. Colocalization was determined by the fluorescence intensity of the protein of interest at each three-dimensional surface compared to the fluorescence intensity of the secondary only control.

**Pull Down:** 10-cm dishes seeded to a final total of  $\sim 5 \times 10^6$  HeLa or 293β5 cells were lysed in a solution of PBS containing 0.1% Triton X-100 and 1mM PMSF for 10 minutes at 4°C. Cells were scraped into a 1.5mL eppendorf, and cell debris pelleted. Supernatant was collected and incubated with NHS-Biotin (final concentration 0.3 mg/ml), 1 hour at 4°C. Simultaneously, 150μg his-tagged pVI or GFP, or a 10% FBS solution alone, was

incubated with 20 $\mu$ l of a 50% cobalt bead resin slurry. Afterwards, the biotinylation reaction was quenched with 40mM glycine (pH 7.5), for 30 minutes at 4°C, and cobalt beads were pelleted at 2000 RPM (1 minute) and washed twice with PBS. Once quenched, lysates were pre-cleared with FBS-incubated cobalt beads plus 10mM imidazole, 1 hour. Preclearing beads were pelleted at 2000 RPM, and supernatant decanted into eppendorfs containing pVI- or GFP-loaded cobalt beads. Lysates incubated at 4°C, 1 hour, then cobalt beads were pelleted and washed twice with PBS, then eluted with 20 $\mu$ l of PBS containing 20, 50, and 500mM imidazole. Bead pellets were resuspended in 20 $\mu$ l of a solution containing 25mM Tris, 25mM NaCl, 0.1mM EDTA, 1% Triton X-100, 0.5% NaDeoxycholate, and 0.1%  $\beta$ -mercaptoethanol. 6x denaturing gel loading dye was added to eluates and beads (to 1x concentration), boiled at 100°C for 5 minutes, pelleted at max speed, and subject to immunoblotting (see below). Blots were probed with streptavidin-HRP solution, 1 hour at room temperature.

**Immunoblotting:** Cells were lysed in a solution containing 25mM Tris, 25mM NaCl, 0.1mM EDTA, 1% Triton X-100, 0.5% NaDeoxycholate, 0.1%  $\beta$ -mercaptoethanol and 1mM PMSF, run on 15% SDS-PAGE gels, and transferred to nitrocellulose membranes (Bio-Rad). Membranes were probed either at 4°C overnight or at room temperature for 1-2 hours in TBST 0.1% with 5% BSA or PBST 0.05% with 5% milk. Mouse and rabbit HRP-conjugated secondary antibodies were used (Thermo Scientific). Membranes were developed with Dura substrate (Thermo Scientific) on GE or Denville film, or using a digital imager.

**Statistical Analysis:** Statistical significance ( $p < 0.05$ ) was assessed using the Student's *t*-test whenever two groups were compared. Data are presented as mean  $\pm$  SEM.

Calculations were performed with Microsoft Excel (Microsoft, Inc.) or GraphPad Prism software (GraphPad Software, Inc.).

## CHAPTER III

### RESULTS

#### **Host autophagic components induced to restrict adenovirus upon endosomal rupture**

##### *Ad-induced membrane rupture recruits LC3 to sites of injury*

Virus induction of autophagy and its effects on replication has been documented for a number of viruses [317-323]. Some of these viruses, such as poliovirus, exploit autophagosome formation to their advantage during replication [318]. Others, like HSV, use virally-encoded proteins to evade autophagic restriction [321]. Studies of autophagy induction in Ad infections is limited to late times in infection, after viral entry and genome delivery occurs, and replication is underway [280, 281, 283]. In these reports, western blot assessment of LC3 cleavage into its lipidated form (LC3-II) suggested Ad-induced autophagy generally occurs at 24 hours post-infect (hpi), and the earliest observed formation of autophagosomes happens at 12 hours post-infection (hpi), based on LC3 cleavage-dependent luciferase secretion assays [283]. However, the earliest time point looked at in these studies was 6 hpi, still well after Ad entry occurs. Furthermore, autophagy incorporation of LC3-II will inevitably turnover the protein upon fusion with lysosomes, thus any LC3 cleaved earlier than 6 hpi is likely degraded prior to the 6 hour readout. Therefore, we sought to assess whether Ad entry stimulates autophagy induction during entry, within 2 hpi.



Human adenovirus 5 (Ad5) with a mutant PPxY motif (Ad5-M1 PPSY to PGAA) in the membrane lytic protein VI (pVI) is ~20-fold defective in infectivity compared to Ad5-WT. Previous data indicate that the mutant retains the capacity to rupture endosomes, as assessed *in vitro* [209], as well as by its ability to induce gal3 punctate staining in immunofluorescence assays (IFA) during infection [210]. However, Ad5-M1 does not efficiently traffic to and accumulate at the perinuclear region of the cell. These observations suggest that this mutant virus's defect lies downstream of uptake and endosomal rupture, but upstream of viral trafficking through the cell cytoplasm. As previous literature indicates that *Salmonella*-mediated vesicle fragmentation induces bacterial sequestration by autophagosomes [72], we hypothesized that Ad does stimulate autophagy upon entry, and that the Ad5-M1 defect is due to enhanced uptake by autophagosomes.

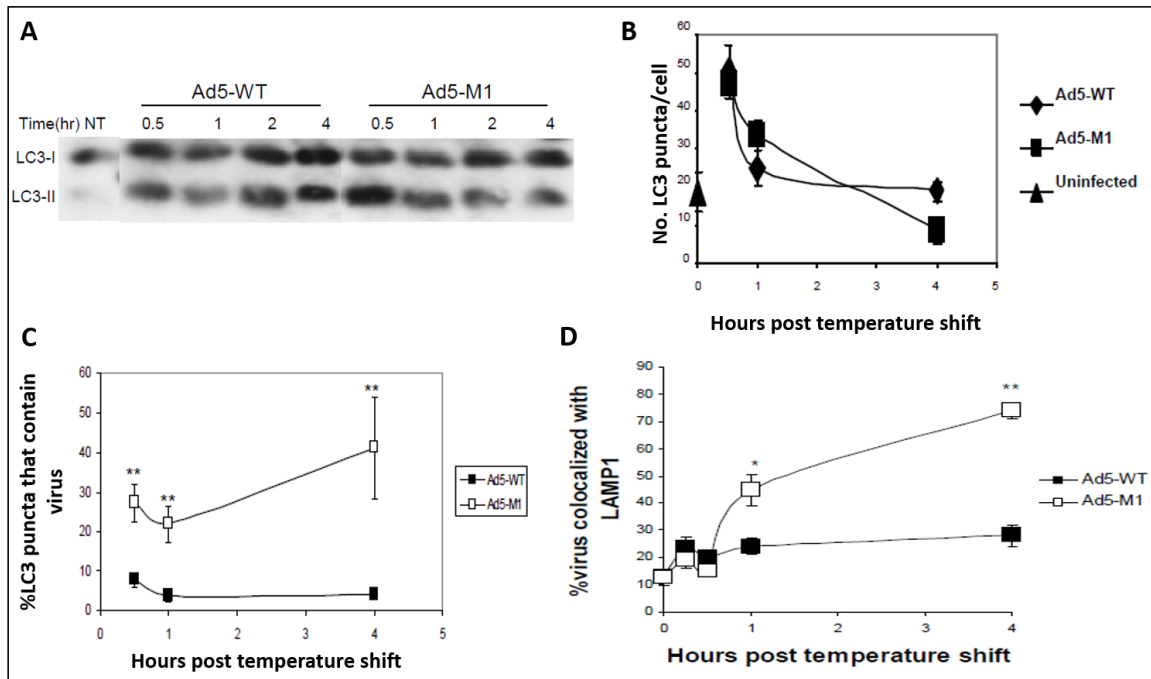
AlexaFluor-488 fluorescently-labeled Ad5-WT or Ad5-M1 virions were incubated with 4°C-chilled HeLa cells on ice to allow virus to bind, but not endocytose into, cells. Once bound, cells were washed with PBS to remove unbound virus, and 37°C pre-warmed media was added to cells to induce synchronous infection. Cells were fixed and stained over a time course to determine whether LC3 is recruited to sites of endosomal rupture. Fixed cells were stained with antibodies against gal3 and LC3 to label ruptured endosomes and autophagosomes, respectively. We found that LC3 is recruited to lysed vesicles within 30 minutes post-temperature shift, and LC3 cleavage during infection was confirmed by western blot (Figure 1A). Both Ad5-WT and Ad5-M1 viruses induce similar numbers of LC3 puncta in cells, but cells transduced with mutant ts1 Ad

that do not rupture endosomes do not induce LC3 punctate staining above background in non-transduced cells. These data suggest that Ad-mediated recruitment of autophagosomes requires membrane rupture (Figure 1B) [324].

*The pVI PPxY motif enhances Ad evasion of host restriction by autophagy*

Although Ad5-WT and Ad5-M1 both induce autophagy, they may not be affected by this host restriction process if they escape from sequestration. It is not known whether these viruses escape from sites of membrane rupture prior to autophagosomal isolation. Since Ad5-M1 is defective for gene delivery, it is conceivable that Ad5-WT, but not Ad5-M1, penetrates into the cytosol in a time-efficient manner, thus evading restriction by autophagosomes. To test this, we assessed whether Ad5-WT efficiently egresses from endosomes prior to LC3 accumulation, compared to Ad5-M1. Using the same time course model described above, we calculated the percent of 488-labeled virus colocalized with LC3. We found that Ad5-M1 is more highly colocalized with LC3 accumulations compared to its WT counterpart when transduced with similar numbers of physical particles per cell, in accordance with our hypothesis (Figure 1C) [324].

The fate of autophagosomal contents is typically degradation after phagophore fusion with lysosomes. However, some pathogens mediate infection by preventing this fusion event. We hypothesized that since Ad5-WT simply evades the initial sequestration event, Ad5-M1 is likely targeted to lysosomes for destruction. We performed a time course to assess virus colocalization with the lysosomal marker LAMP1. We found that, compared to Ad5-WT, Ad5-M1 is significantly more associated with LAMP1-positive membranes, suggesting that the mutant virus is more efficiently targeted to degradative



**Figure 1: Effects of autophagy in Ad restriction during entry.** HeLa cells infected with AlexaFluor 488-labeled Ad5-WT or Ad5-M1 virus were either lysed or fixed. **A.** Western blot of whole cell lysates probed for LC3. **B.** Quantification of the number of LC3 puncta per cell. **C.** LC3 puncta colocalized with Ad5. **D.** Ad5 colocalization with LAMP1. \* $p < 0.05$ , \*\* $p < 0.001$

lysosomes after appropriation by autophagosomes (Figure 1D). Taken together, these data indicate that Ad-induced membrane damage mediates LC3 conscription to damaged vesicles, and the pVI-PPxY motif is critical for the virus to efficiently abscond from these sites to evade phagophore sequestration. The concomitant destruction of autophagosomal contents by lysosomes is the mechanism by which the cell restricts viral particles unable to escape from ruptured endosomes. This correlates with previous findings that viral genomes are quickly degraded after entry in Ad5-M1 transductions, but not for Ad5-WT [325]. Additionally, knocking down components of the autophagy expansion process restores Ad5-M1 infectivity to wild-type levels, further demonstrating that autophagy is a critical mechanism employed by the cell to restrict incoming viruses upon exposure to the cytoplasm [324].

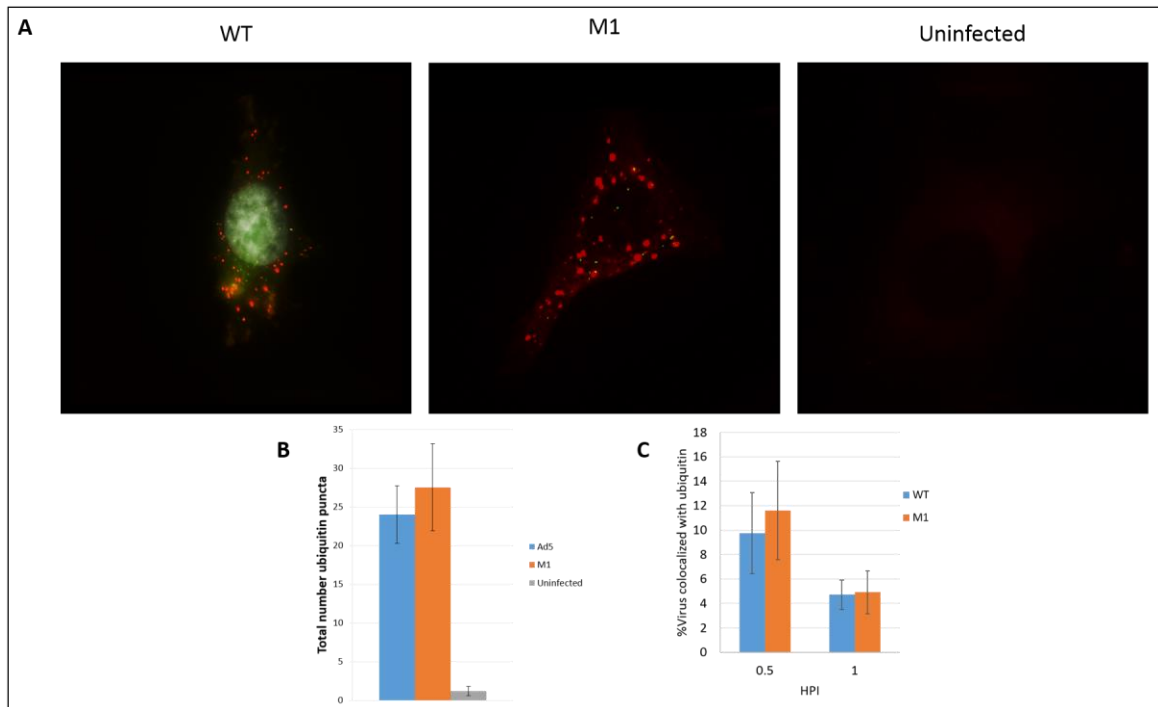
#### *TBK1 activity recruits LC3 to Ad-ruptured endosomes*

The previous results show that LC3 is recruited to Ad5-ruptured endosomes and that autophagy restricts Ad5-M1 infection, however these experiments do not reveal mechanism of autophagy activation by Ad cell entry. We sought to determine the host molecules involved in activating autophagy and completing phagophore formation. Other data demonstrate that during infection with intracellular bacterial pathogens, such as *Salmonella* and *Shigella*, membrane rupture promotes cell-mediated ubiquitination of the pathogens, which is important to efficiently target autophagy machinery to sites of damage [76, 85]. Pathogen ubiquitination facilitates the conscription of ubiquitin-binding adaptor proteins p62, NDP52, and optineurin (OPTN) to sites of vesicle fragmentation

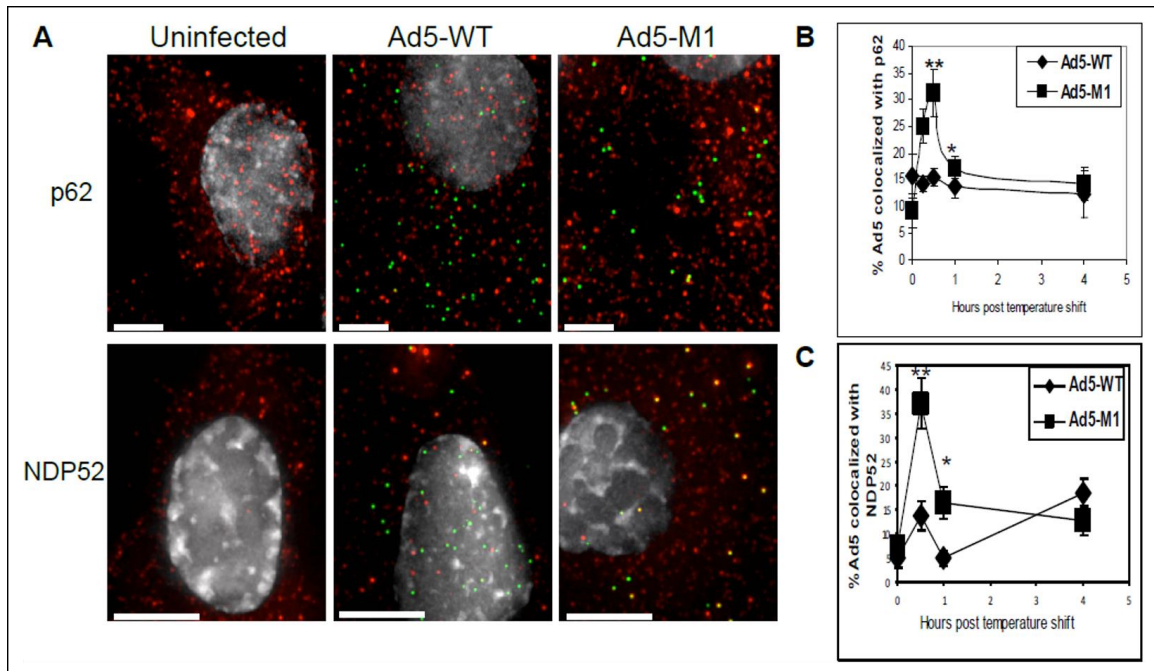
during infection [81, 82, 326]. NDP52, p62, and OPTN in turn are part of a danger signaling pathway to target cargo for degradation via autophagy.

*In vitro* biochemical analyses suggest that pVI is ubiquitinated by host Ub ligases in a PPxY-dependent manner [209]. We asked whether the PPxY motif mediates ubiquitin targeting to sites of membrane rupture in the context of infection. Preliminary IFA studies suggest that both Ad5-WT and Ad5-M1 induce similar numbers of ubiquitin puncta upon rupture (Figure 2). Additionally, there is no difference in the percent of virions colocalized with ubiquitin with either virus, suggesting that the pVI-PPxY motif does not specifically mediate ubiquitin targeting to sites of membrane damage. This result is in agreement with previous reports indicating that membrane rupture mediated by transfecting cells with latex beads is sufficient to conjugate ubiquitin to host proteins [77]. However, our IFA data do not specify whether the virion itself or host proteins are ubiquitinated. Taking our findings with the *in vitro* data previously described, perhaps the PPxY motif is important to properly ubiquitinate pVI, but has no effect on the ubiquitination status of neighboring host proteins.

Next, we assessed whether ubiquitination at sites of rupture recruits ubiquitin-binding adaptor molecules in a pVI-PPxY-dependent manner. IFA assessment of p62 and NDP52 location within the cell shows that Ad infection induces an accumulation of these proteins during entry, and Ad5-M1 colocalizes with them significantly more than Ad5-WT, with kinetics similar to those seen with gal3 colocalization (Figure 3) [324]. These results indicate that, akin to what is seen during bacterial-induced membrane rupture, p62 and NDP52 recruitment occur upon endosomal disruption by Ad.



**Figure 2: Ad5 colocalization with ubiquitin.** HeLa cells infected with AlexaFluor 488-labeled Ad5-WT or Ad5-M1 virus (green) were fixed and stained for ubiquitin (red) one hour post-entry. **A.** Representative micrographs of Ad5-WT, Ad5-M1, or uninfected cells. Virus (green). **B.** Quantification of the number of ubiquitin puncta per cell at one hour post-infection. **C.** Percent of virus colocalized with ubiquitin puncta.

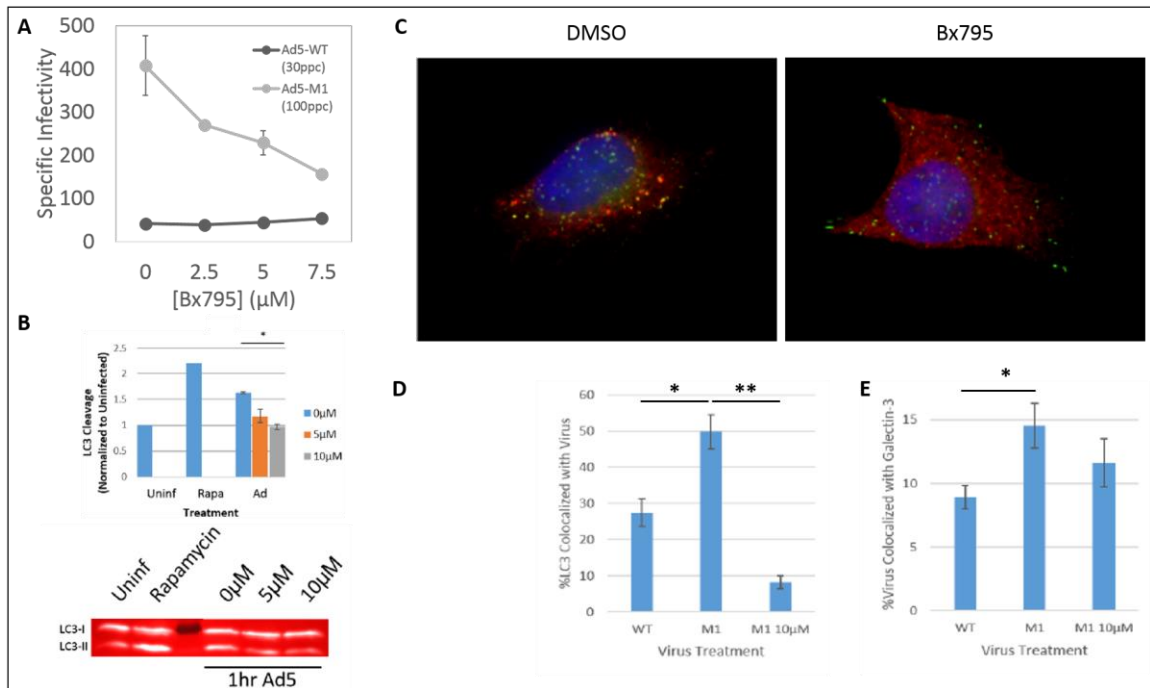


**Figure 3: Ad5 colocalization with adaptor proteins p62 and NDP52.** HeLa cells infected with Ad5-WT or Ad5-M1 (green) were fixed and stained for p62 or NDP52 (red). **A.** Representative micrographs from 30 minutes post-infection. Bar is 5 μm. **B.** Ad5 colocalization with p62. **C.** Ad5 colocalization with NDP52. \* $p < 0.05$ , \*\* $p < 0.001$

Once NDP52 binds ubiquitinated *Salmonella* after membrane damage, the adaptor molecule complexes with the protein kinase TANK-binding kinase-1 (TBK1), which in turn phosphorylates NDP52, p62, and OPTN molecules bound to ubiquitin to label them and their bound targets as autophagic cargo [81, 82, 326]. Phosphorylation by TBK1 increases the affinity of these adaptors for LC3, which is involved in elongation of the autophagic phagophore. We next asked whether TBK1 activity is similarly important in restricting Ad infection during entry. A reversible chemical inhibitor of TBK1, Bx795, acts as an ATP competitive inhibitor to impede TBK1's phosphorylating kinase activity [327]. Bx795 prevents OPTN phosphorylation, which is necessary for restricting *Salmonella* activity [81].

To test whether a similar occurrence happens during Ad infection, we pre-treated cells with various amounts of Bx795, ranging from 2.5 $\mu$ M to 7.5 $\mu$ M, or vehicle control (0.1% DMSO) for 30 minutes. Cells were transduced with replication-defective Ad5-WT or Ad5-M1 viruses containing a CMV-driven GFP transgene in the presence of the same concentration of drug during pre-treatment (Ad5-WTgfp and Ad5-M1gfp, respectively), for two hours. Finally, cells were washed three times and treated for one more hour with drug or vehicle before being washed three times with PBS and fed complete medium without drug. Cells were incubated overnight, between 18-24 hours to ensure genome delivery, and GFP expression was assessed by flow cytometry. We found that inhibiting TBK1 phosphorylation activity with Bx795 enhances Ad5-M1 infectivity in a dose-dependent manner in transduction assays (Figure 4A). These results suggest to us that one or more adaptor molecules is necessary for efficient restriction of Ad5-M1 infection.





**Figure 4: TBK1 activity in autophagosomal restriction of Ad5.** **A.** HeLa cells treated with varying doses of Bx795 were transduced with Ad5-WT or Ad5-M1. Specific infectivity (particles per GFP-transducing unit, or GTU) is calculated as the number of viral particles added to a well divided by the total GFP-positive cells in the well. **B.** Whole cell lysates infected with Ad5 in the presence of 0, 5, or 10 $\mu$ M Bx795 were probed for LC3 cleavage by western blot. Uninfected and rapamycin lanes serve as positive and negative controls, respectively. **C.** Representative micrographs of HeLa cells infected with 488-labeled Ad5-M1 (green) and stained for LC3 (red) in the presence or absence of 10 $\mu$ M Bx795 one hour post-infection. **D.** LC3 puncta colocalized with virus. **E.** Percent of Ad5 colocalized with galectin-3. \* $p$ <0.05, \*\* $p$ <0.001

Previous reports indicate that Bx795 treatment has no effect on starvation-induced LC3 cleavage in MEFs, so we hypothesized that Bx795 would have no effect on LC3 cleavage in our assay [83]. Cells were synchronously infected with either Ad5-WT or Ad5-M1 in the presence or absence of 10 $\mu$ M Bx795. Thirty minutes post-warming, cells were trypsinized, lysed, and subjected to western blot procedures. Cleavage and lipidation of LC3-I results in a lower molecular weight band when probed with anti-LC3 antibodies (LC3-II). Thus, lysates were probed for LC3, and the amount of LC3-I converted to LC3-II was assessed by densitometry as a percentage of total LC3. Contrary to our expectations, we found that treating cells with Bx795 diminishes the amount of LC3-II formed during infection (Figure 4B) compared to DMSO-treated transduced and rapamycin controls. In fact, 10 $\mu$ M Bx785 treatment reduces LC3 cleavage to background levels. Although this result is surprising, it implies that in HeLa cells, TBK1 activity is important for LC3 cleavage during viral transduction, further confirming its importance in restriction during Ad entry.

Since Ad5-M1 infectivity is enhanced when cells are treated with Bx795, and LC3 cleavage is reduced, it stands to reason that Ad5-M1 would not associate with LC3 during the course of infection. However, even in the absence of LC3, the mutant virus should still be defective in timely endosomal escape. Thus, we hypothesized that cells infected with Ad5-M1 in the presence of Bx795 will lead to a reduction in virus colocalization with LC3, but virus should remain associated with ruptured endosomes at late times during entry. To test this, IFA was performed on cells pre-treated with 10 $\mu$ M Bx795 or 0.1% DMSO. Cells were transduced with 488-labeled Ad5-WT in DMSO or

Ad5-M1 in either DMSO or Bx795, and fixed two hours post-warming. Fixed cells were stained for gal3 and LC3, and colocalization assessed. We found that at 1 hour post-infection, Ad5-M1 virus in cells treated with Bx795 associates with autophagosomes significantly less often compared to vehicle control treated, compared to vehicle-control transduced cells (Figure 4D). Ad5-M1 colocalization with gal3 is not statistically significantly different between Bx795 treatment and DMSO controls (Figure 4E). These data indicate that, similar to the responses induced during intracellular bacterial infections, autophagy induction during endosomal rupture by Ads requires TBK1 activity to expand and complete autophagosomes formation to sequester endosome-associated contents. Furthermore, Ad5-M1's inability to escape restriction by autophagy occurs due to an inability to egress away from endosomal fragments before autophagosomal uptake.

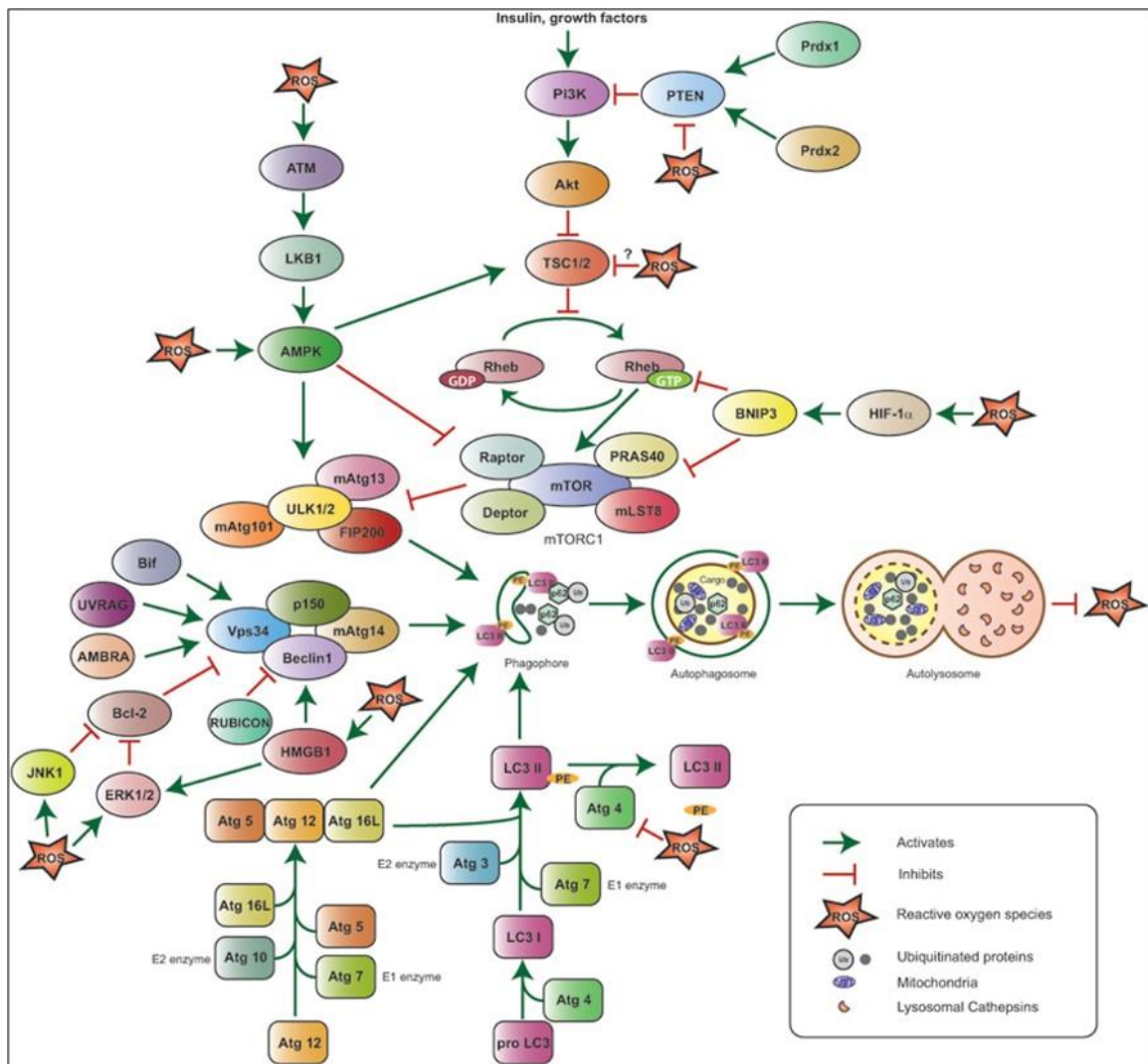
*Autophagy signaling pathways assessed during Ad infection*

To this point, we have assessed the mechanisms of autophagic restriction of Ad during entry. However, we have not examined the method of autophagy induction after endosomal lysis. Much interest lies in how autophagy is induced and subsequently recruited to Ad-ruptured endosomes, for both developing treatments against Ad infection as well as designing improved Ad vectors for vaccine development. Our lab previously showed that knocking down gal8 with siRNA enhances Ad5-M1 infectivity to Ad5-WT levels, corresponding to reduced Ad5-M1 colocalization with LC3-positive autophagosomes. However, autophagosome formation is still induced to similar levels when compared to control siRNA cells. These results implicate that membrane rupture alone is sufficient to induce autophagy, but the cell cannot properly target

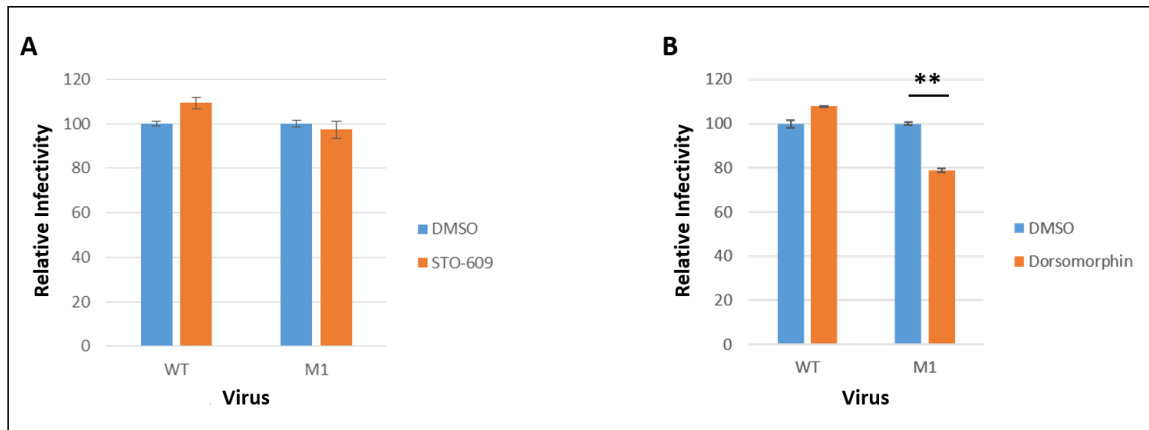
autophagosomes to sites of injury in the absence of gal8. To this end, we sought to determine the pathway by which Ad entry induces autophagy.

Many pathways feed into autophagosome formation, each involving various signaling components (Figure 5). Data from the laboratory of Harald Wodrich at the University of Bordeaux suggest that, while the canonical pathway of autophagy activation through mTOR inhibition is regulated by Ad during infection, mTOR's function is dispensable regarding host restriction of Ad5-M1 in U2OS cells (personal communication). We also assessed some of the pathways that feed into autophagy activation through the mTOR pathway. We used inhibitors of upstream signaling molecules of mTOR, 5' AMP-activated protein kinase (AMPK, dorsomorphin) and calcium/calmodulin-dependent protein kinase kinase 2 (CaMKK2, STO-609), to assess whether they are important for restricting Ad through autophagy during infection. Signaling through either of these molecules activates autophagy by inhibiting mTOR function, so if mTOR inactivation via AMPK or CaMKK2 is important to restrict Ad infectivity, then treating cells with these inhibitors should restore Ad5-M1 infectivity to WT levels. We observed little change in the specific infectivities of either Ad5-M1 gfp or Ad5-WTgfp when treated with these inhibitors (Figure 6), corroborating the evidence from Dr. Wodrich's lab. Taken together, these data would suggest that autophagy induction occurs in an mTOR-independent pathway.

We previously reported that Ad-induced membrane damage mediates the production of high levels of reactive oxygen species (ROS) [31, 329]. ROS acts as an immune signal of infection and cell stress to activate the NLRP3 inflammasome, and



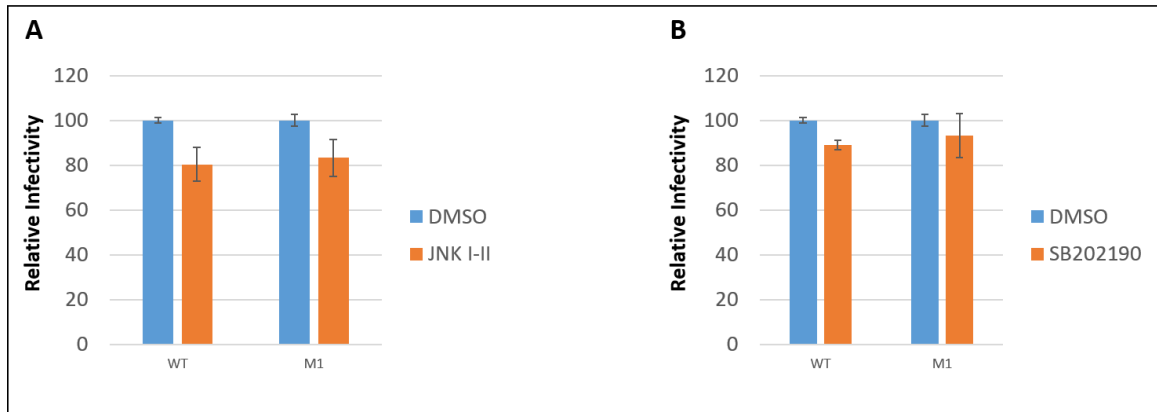
**Figure 5: Signaling pathways mediating autophagy regulation.** Adapted from [328]. Multiple signaling cascades, including mTOR-dependent and –independent pathways, have a described capacity to induce autophagosome formation via initiation by unique stimuli.



**Figure 6: AMPK signaling during Ad5 entry.** HeLa cells were transduced with Ad5-WT or Ad5-M1 in the presence of 0.1% DMSO vehicle, 1 $\mu$ g/ml STO-609 (**A**), or 40 $\mu$ M dorsomorphin (**B**). Results displayed as infectivity relative to vehicle control set to 100%. \*\*p<0.001

augments TLR9-dependent cytokine expression. In addition to this role, others have demonstrated a complex role for ROS in signaling the induction of autophagy [330-332]. These reports, in addition to our observations that membrane rupture induces both ROS and autophagosome formation, suggest that ROS produced upon Ad transduction may signal autophagy induction.

Our lab previously showed that siRNA knockdown of beclin-1 partially restores Ad5-M1 infectivity. ROS signaling feeds into beclin-1 activation, which is mediated by JNK1 and ERK1/2 activation. ROS activates JNK1 and ERK1/2, which both indirectly activate beclin-1 by inhibiting Bcl-2, a beclin-1 inhibitor. Unpublished results from our lab suggest that chemical inhibition of ERK using PD90859 has little effect on general Ad5 infectivity (data not shown). Similarly, we chemically inhibited JNK1 to test whether this pathway is involved in restricting Ad5-M1 infectivity. In our hands, inhibiting JNK1 with JNK inhibitor I-II results in insignificant decreases in the infectivities of both Ad5-WTgfp and Ad5-M1gfp (Figure 7A), suggesting that beclin-1-facilitated autophagy induction is not mediated by these molecules during Ad entry. Very recently, p38 mitogen-activated kinase (p38 MAPK, or p38) was observed to activate autophagy in CD8<sup>+</sup> cells in an mTOR-independent pathway [333]. Others have shown that p38 function is important in mediating ROS-dependent activation of autophagy-related genes [334]. Although limited reports suggest that IFN $\gamma$ -induced autophagy is mediated by p38 signaling [335, 336], no one to our knowledge has assessed whether membrane damage activates autophagy via p38 signaling. We tested whether this is the case during Ad infection using a potent and specific inhibitor of p38, SB202190, in



**Figure 7: ROS-dependent beclin-1 activation in Ad5 infection.** HeLa cells were transduced with Ad5-WT or Ad5-M1 in the presence of 0.1% DMSO vehicle, 10 $\mu$ M JNK I-II (A), or 20 $\mu$ M SB202190 (B). Results displayed as infectivity relative to vehicle control set to 100%.



transduction assays with Ad5-WTgfp and Ad5-M1gfp. However, we found that the inhibitor has little effect on viral infectivity for either virus, suggesting that p38-dependent activation of autophagy is not responsible for induction by Ad entry (Figure 7B).

Nod1 and Nod2 receptors have a role in restricting bacterial infection by recruiting autophagy proteins to the site of entry [70, 337]. Furthermore, Nod2-dependent responses were previously observed to contribute to proinflammatory responses against helper-dependent adenoviral vectors in a MyD88-independent manner [338]. We assessed whether either of these proteins are important in Ad induction of autophagy. Using siRNA to knock down each protein, we assessed the infectivity of Ad5-WTgfp and Ad5-M1gfp viruses compared to control siRNA cells. We found that knocking down Nod2 has no effect on virus infectivity, however knocking down Nod1 significantly increases the percent of GFP-positive cells for both viruses (Figure 8). These data suggest that Nod1, but not Nod2, is important in restricting Ad infection, but in a pVI-PPxY-independent manner. This is in agreement with previously published results. However, we did not further assess Nod1's function in Ad entry.

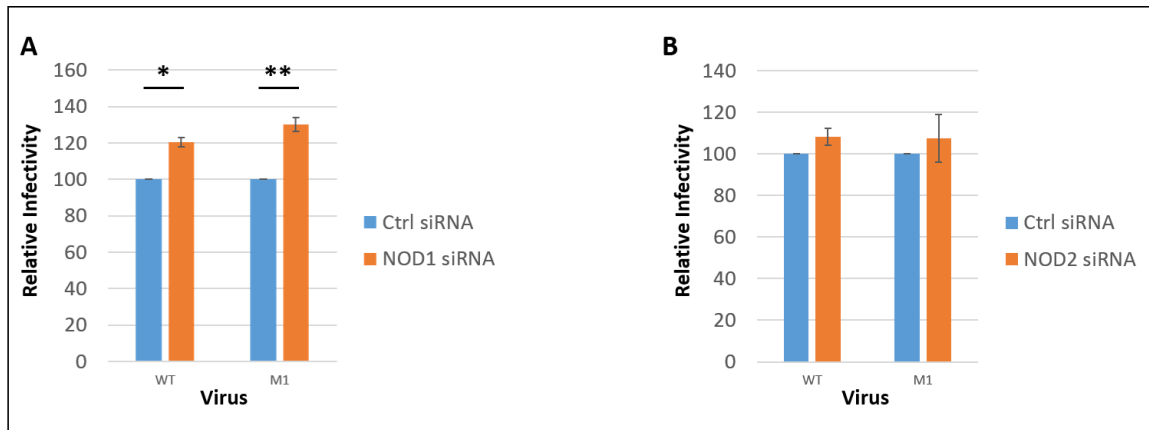
Taken together, we show that autophagy is a major factor in restricting Ad5-M1 infection during viral entry. Recruitment of ubiquitin-LC3 adaptor molecules and their phosphorylation by TBK1 is critical to sequester and limit the mutant virus. We also found that the JNK1 activity may have a role in efficient Ad infection, but occurs in a pVI-PPxY-independent manner. A recent report indicates that in airway epithelial cells, starvation-induced autophagy aids Ad infection [339]; perhaps initiation of the

autophagic machinery involving this pathway is important to recruit some host factor or factors necessary for Ad endosomal escape or trafficking through the cytosol. On the other hand, siRNA knockdown of Nod1 increases infectivity of both viruses, suggesting that Nod1 plays an uncharacterized role in restricting Ad entry. How this occurs, and how autophagy is induced and restricts Ad in a pVI-PPxY-dependent manner, still requires testing.

### **Contributions of dynein motor function during Ad entry**

As suggested above, Ad must evade autophagy to efficiently infect cells. To do so, we hypothesize that Ad egresses from endosomes, penetrating into the cytosol and away from the autophagy machinery. The current body of literature suggests that viral trafficking throughout the cell requires transport on microtubules, utilizing the microtubule motors dynein and kinesin. We therefore asked whether one or both of these motor proteins is important in facilitating Ad evasion of autophagy.

During the initial viral uncoating process, pVI releases from the capsid and ruptures endosomes, exposing the virion to the cytoplasm [206, 208, 210]. Once the virus has escaped into the cytosol, pVI does not leave with the capsid, but remains associated with the endosomal fragments. Depolymerizing microtubules using nocodazole significantly restricts Ad5-WT infectivity, but has no effect of Ad5-M1, suggesting that a failure of pVI to associate with microtubules, either directly or indirectly, causes the mutant virus' defect. Wodrich, et al. found that transient overexpression of RFP-tagged pVI-WT highly colocalizes with tubulin, but similar experiments using pVI-M1 show very little association with tubulin [209]. Although no literature to date has suggested pVI



**Figure 8: Contributions of NOD signaling during Ad5 infection.** HeLa cells knocked down for NOD1 (A) or NOD2 (B) via siRNA were transduced with Ad5-WT or Ad5-M1. Results displayed as infectivity relative to siRNA control set to 100%. \* $p < 0.05$ , \*\* $p < 0.001$

to specifically interact with microtubules or microtubule-associated proteins, these data imply a role for pVI in microtubule interactions. Recent studies show that the majority of incoming virions colocalize with dynein heavy chain in IFA experiments, as well as a high association with dynactin subunits [290]. They also show that adenovirus capsids only colocalize with DHC once they have egressed from clathrin-positive or EEA1-positive locations, and immunoprecipitations using anti-hexon antibodies pull down dynein intermediate chain components 40 minutes post-infection. *In vitro* assessments imply that hexon directly associates with dynein, however this occurs in a pH-dependent manner, requiring a pH shift to at least 5.5. Since virus ruptures endosomes at very early times, this suggests that the hexon-dynein interaction is not physiologically relevant, at least from a pH-dependent context. These contradictions merit further study into how Ad exploits microtubule motor proteins, and what each motor's individual contributions are during entry.

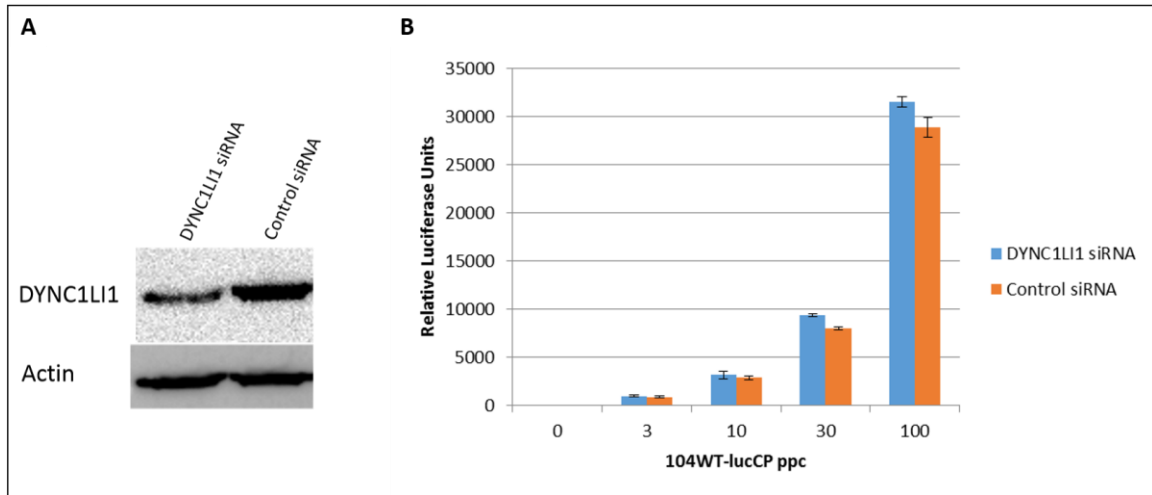
*DLC1 interactions with hexon are not physiologically relevant*

Previous reports indicate that light intermediate chain 1 (LIC1) is a critical component in recruiting Ad virions to dynein during infection [290]. Moreover, PKA-phosphorylated LIC1 is necessary to efficiently translocate virus to the MTOC or perinuclear area 1 hpi [340]. However, the effect on viral infectivity was not assessed. Using siRNA knock downs, we asked how LIC1 specifically contributes to Ad infectivity during the entry process. We expected that if LIC1 is important for Ad accumulation near the nucleus, then knocking down LIC1 would restrict Ad infectivity. HeLa cells were transfected twice with siRNA against LIC1 or a control siRNA once every 24 hours.

Another 24 hours after the second transfection, cells were lifted and either saved for western blot or plated, rested, and transduced with a replication-defective Ad5 vector containing a CMV-driven luciferase transgene overnight. Approximately 15 hours after transduction, cells were lysed, and lysates assessed for luciferase activity. We found that LIC1 siRNA transfection reduced LIC1 levels in the cell by just over 40% (Figure 9). However, when assessing luciferase expression, we found little difference in infectivity between LIC1 and control knock down cells. This result suggests that although LIC1 interacts with pH-primed hexon *in vitro* and non-phosphorylated LIC1 reduces virus accumulation at the nucleus an hour after entry, LIC1 is overall unimportant for Ad genome delivery.

*Dynein inhibition by Ciliobrevin D restricts WT Ad to endosomes during entry*

Although LIC1 appears unimportant for virus infectivity, a large body of literature still indicates that dynein function is critical during Ad entry. To elucidate the specific contributions of dynein on Ad endosomal escape, we utilized a recently developed specific small molecule inhibitor of the motor protein dynein, called Ciliobrevin D (CilioD). Ciliobrevins are a group recently designed small molecules that have nanomolar affinities for the dynein ATPase pocket of the DHC motor [341]. These are specific for dynein motors, as *in vitro* assays show they elicit no effect on kinesin-1 or -5 movement along microtubules. Others showed that inhibition mediated by these molecules is reversible in neuronal cell cultures [342, 343]. These results illustrate the usefulness of ciliobrevins in future studies to elucidate the contributions of dynein during infection at specific times without requiring constitutive dynein inhibition through siRNA



**Figure 9: Effects of dynein LIC1 knockdown on Ad5 infectivity.** DLC1 was knocked down in HeLa cells via siRNA, and cells were transduced with Ad5WT-luc virus. **A.** Western blot of whole cell lysates probed for DLC1 (DYNC1LI1). **B.** Luciferase expression in DLC1 and control knockdown cells transduced with increasing viral particles per cell (ppc).

knockdown or dominant negative overexpression models, minimizing the repercussions one sees when shutting down a critical transport pathway within the cell.

We expected that inhibiting dynein with Ciliobrevin D (CilioD) would restrict Ad5-WT infectivity, in accordance with previous reports using dynein knock down or constitutive inhibition. Cells were pretreated with 50 $\mu$ M or 100 $\mu$ M concentrations of drug or 0.2% DMSO as a control for 30 minutes, and similarly infected as described above for Bx795 treatments. We found that transducing cells with Ad5-WTgfp in the presence of CilioD significantly restricts infectivity by more than 60% using 100 $\mu$ M concentrations of the drug (Figure 10A). We additionally anticipated that since pVI-M1 does not colocalize with microtubules and Ad5-M1 cannot efficiently egress from endosomes, treatment with CilioD would have no effect of Ad5-M1 infectivity. To our surprise, we saw that CilioD enhances Ad5-M1gfp infectivity by 50% (Figure 10A). In fact, treating cells with 100 $\mu$ M CilioD equilibrated Ad5-WT and Ad5-M1 infectivities when cells were transduced with the same physical particles per cell. This was intriguing to us, suggesting that not only is Ad transport intimately linked to dynein for infectivity, but so is host-mediated restriction of Ad5-M1. Lysosomal and late endosomal movements throughout the cell's cytoplasm is dependent on both dynein and kinesin associations [344-346]. Inhibiting dynein likely prevents lysosomes and/or autophagy machinery from translocating to Ad5-containing endosomes after lysis, thus giving Ad5-M1 as much of an opportunity to infect a cell as the WT virus in the absence of dynein.

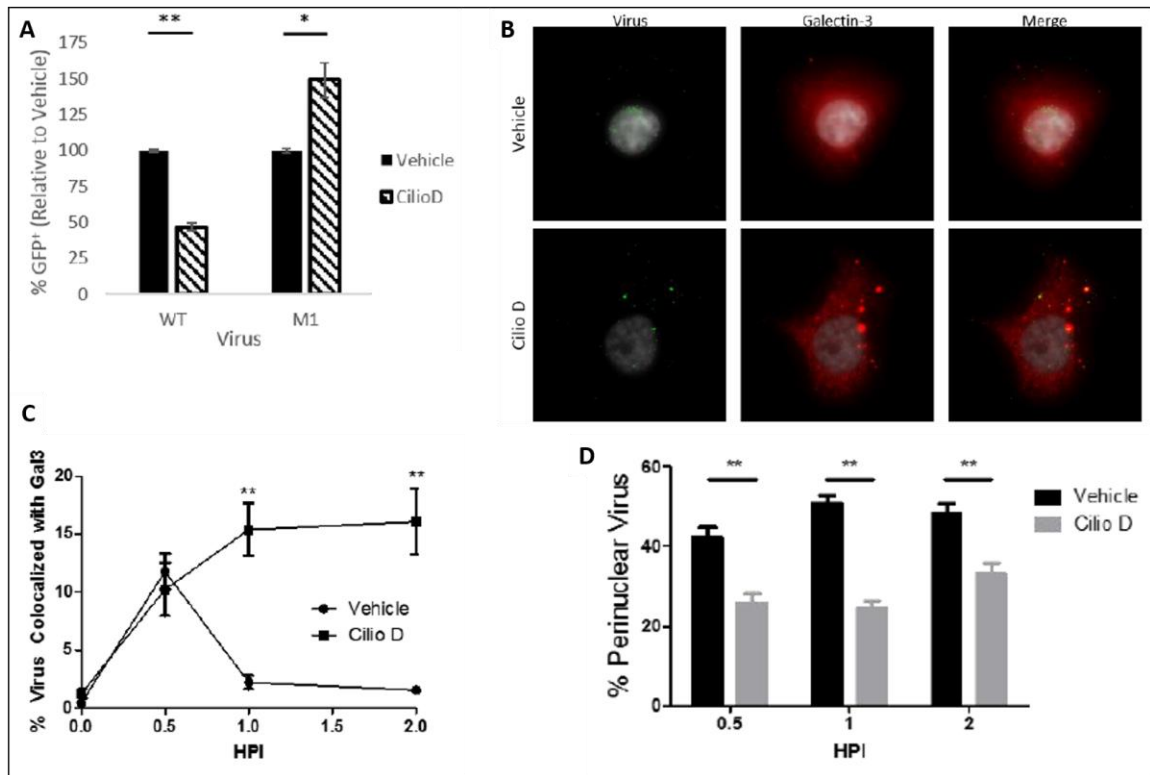
We wanted to further characterize the defect caused by dynein inhibition by assessing Ad5-WT kinetics in the cell during entry. We expected that infections with the

Ad5-WT in the presence of CilioD would recapitulate the phenotype we see with our Ad5-M1 virus: reduced accumulation at the perinuclear area and increased colocalization with gal3 endosomes. We utilized IFA to evaluate Ad5 transport within the cell, as well as its colocalization with gal3-positive endosomes, over the course of a 2 hour transduction. Cells were chilled on ice at 4°C in the presence of drug or vehicle control, 30 minutes. Chilled virus + treatment was added to chilled cells, on hour, allowing virus to bind, but not endocytose. After binding, cells were washed three times with ice-cold PBS before being fed pre-warmed 37°C medium containing drug or vehicle. Cells were fixed and stained for gal3 and LC3, and virus localization was assessed. We saw that treatment with this drug leads to greater colocalization of Ad5-WT with gal3, implicating dynein's significance in endosomal escape (Figure 10C). In addition to increased colocalization with ruptured endosomes, fewer virions accumulate at the perinuclear area over the course of two hours, further implicating dynein's importance for proper Ad entry (Figure 10D). These data implicate some mechanism of dynein recruitment to endosomes that is dependent on the pVI-PPxY motif.

*Adenovirus infection requires BAG3 in a PPxY-independent manner*

Understanding what host proteins interact with the pVI-PPxY domain will lead us to further understand the mechanism by which incoming virions utilize microtubules to escape from endosomes. *In vitro* studies show that pVI interacts with a number of Nedd4-family E3 ubiquitin ligases in a PPxY-independent manner [209]. These ubiquitin ligases contain a protein-protein interacting WW-domain sequence motif that recognize proline-rich sequences, including PPxY motifs, in host proteins [347, 348]. Once engaged with a

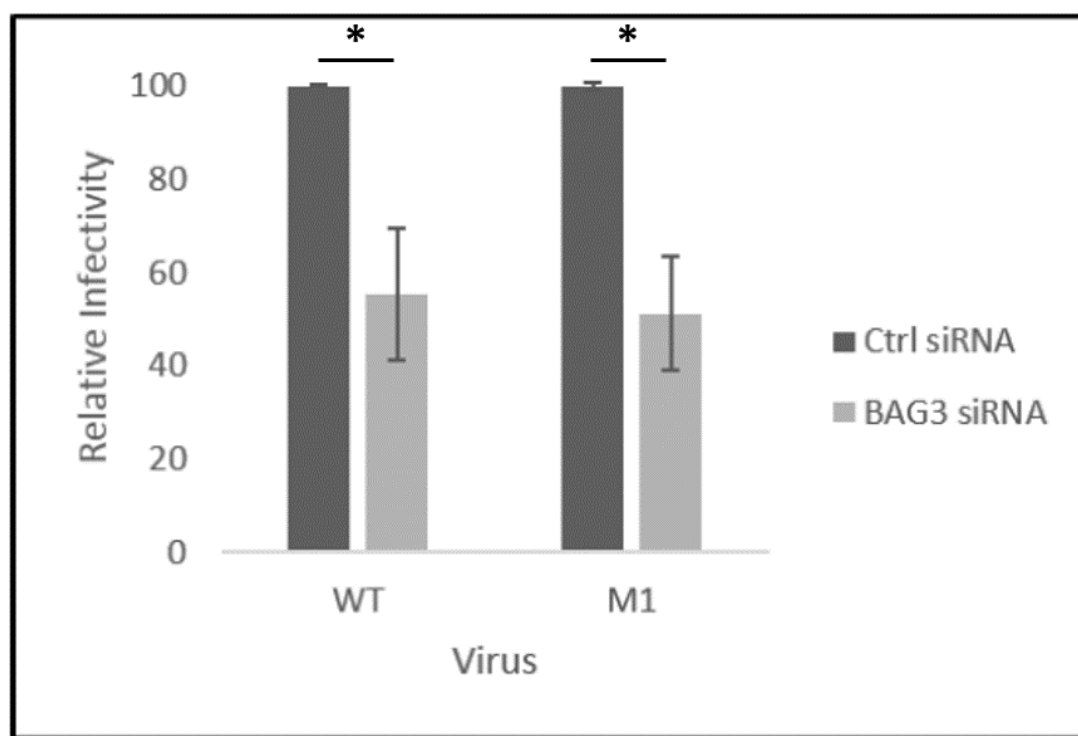




**Figure 10: Changes in Ad5 localization upon dynein inhibition.** **A.** HeLa cells treated with 50 $\mu$ M CilioD were transduced with Ad5-WT or Ad5-M1. Infectivity is displayed relative to DMSO vehicle treatments, set to 100%. **B.** Representative micrographs of cells transduced with 488-labeled Ad5-WT virus (green) in the presence or absence of CilioD. Cells were fixed and stained for galectin-3 (red). **C.** Percent of virus colocalized with galectin-3 over time. **D.** Percent of virus targeted to the perinuclear area (within 2 $\mu$ m of the nucleus) in the presence or absence of CilioD. \* $p$ <0.05, \*\* $p$ <0.001

protein, the ubiquitin ligase ubiquitinates both itself proteins in close proximity, generally targeting them to the proteasome for degradation [349]. Of these Nedd4 family ligases, Nedd4.2, but not Nedd4.1 or AIP4, depletion results in a modest restriction of Ad5 infectivity that incompletely recapitulates the Ad5-M1 phenotype [209]. Thus, other WW-domain proteins capable of interacting with pVI via the PPxY sequence may be important to evade autophagy.

BAG3 is a WW-containing protein part of a protein complex that facilitates the recognition of cargo targeted for autophagy [350, 351]. BAG3, along with E3-ubiquitin ligases, recruits ubiquitinated p62 and other adaptor proteins to bind cleaved LC3 and expand the autophagosome. Previous studies report that BAG3 knock down attenuates viral transgene expression when cells are infected with replication-defective Ad [352]. Since we observe that pVI remains within fragmented endosomes during Ad egress, we hypothesized that the pVI-PPxY motif recruits BAG3 complexes to ruptured endosomes, mediating Ad escape into the cytosol. To test this, we used siRNA to knock down BAG3 in HeLa cells. After two transfections, cells were used for western blot or plated for transduction. BAG3 knock down or control-transfected cells were transduced with either Ad5-WTgfp or Ad5-M1gfp, and GFP expression was assessed 18-24 hours later. We expected that if pVI-PPxY facilitates BAG3 recruitment to ruptured endosomes to aid in virus escape, then Ad5-WT infectivity should be restricted to Ad5-M1 levels. However, we found that BAG3 knockdown restricts both Ad5-WT and Ad5-M1 infectivities to similar extents (Figure 11). This result suggests to us that, while BAG3 is critical for optimal Ad infection, the pVI-PPxY motif does not contribute to this effect. As



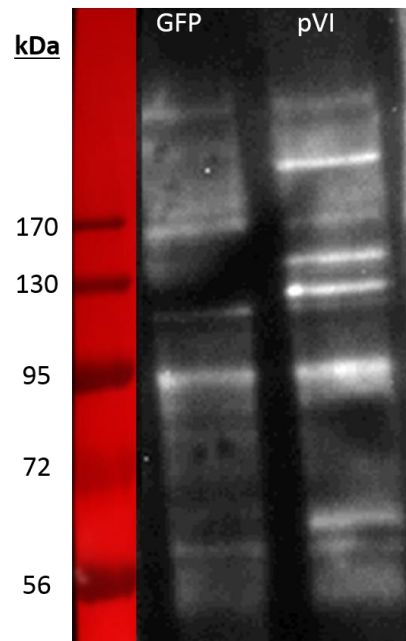
**Figure 11: Ad5 infectivity in BAG3 knockdown cells.** HeLa cells were transduced twice with BAG3 or control siRNA. 72 hours post initial transfection, cells were transduced with Ad5-WT or Ad5-M1. Results displayed as infectivity relative to control siRNA set to 100%. \* $p < 0.05$ , \*\* $p < 0.001$

discussed, perhaps BAG3 knockdown reduces autophagy induction below the threshold required for optimal Ad infection. Alternatively, previous studies show that penton base overexpression in cells results in BAG3 accumulation at the nucleus, and this occurs in a PPxY-dependent manner. Although penton base is shed from the capsid early upon endocytosis, perhaps it mediates an important interaction with BAG3 to facilitate optimal infection.

*In vitro pVI-WT pull downs suggest multi-protein interactions*

Overexpression and pull down data indicate that, in addition to Nedd4-family ligases, pVI interacts with microtubules in a PPxY-dependent manner. Whether MT association is direct or indirect is still undetermined. To further probe potential host proteins interacting with pVI, we performed pull downs using His-tagged pVI-WT. HeLa cells were lysed and cell debris pelleted, and supernatants were biotinylated. After biotinylation, lysates were incubated with purified pVI-his protein or GFP-his as a control. Protein complexes were pulled down with his-tag-binding cobalt beads, washed with imidazole, subjected to western blot, and probed for biotinylated proteins.

We found that, compared to background pull down protein interactions with GFP, a number of bands of various molecular weights are pulled down by pVI-WT (Figure 12). Some of these bands potentially correspond to dynein components, so we repeated the pull down with non-biotinylated lysates, and probed directly for dynein components. Unfortunately, the components we probed for either pulled down with both pVI and GFP incubations, or did not pull down at all (data not shown). We were unable to perform similar pull downs with pVI-M1, as we could not purify sufficient quantities of



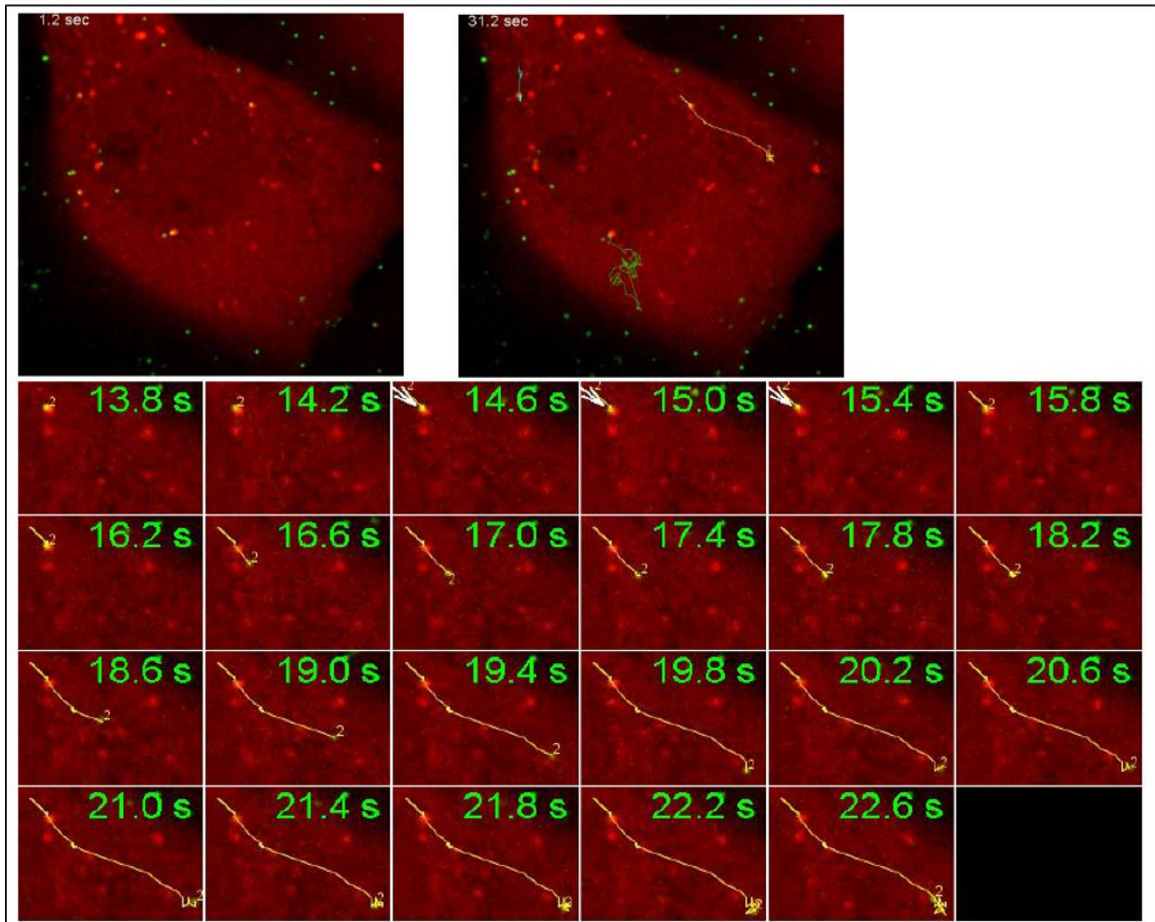
**Figure 12: Protein interactions with pVI via pull down.** Cell lysates were biotinylated, quenched, and incubated with bacterially-derived recombinant HA-tagged pVI or GFP. Interacting proteins were pulled down via cobalt beads, washed, and eluted with imidazole. Eluates were run on western blot and probed with avidin-HRP.

his-tagged pVI-M1. Regardless, these data indicate that pVI specifically mediates a variety of interactions with host proteins. Further elucidation will likely reveal more information regarding these interactions.

### **Influences of kinesin and capsid-associated protein IX during endosomal escape**

We next sought to elucidate the specific contributions of kinesin in Ad infection. A recent report implicates pIX-kinesin interactions for genome delivery [291]. The data suggest that pIX interacts with the nuclear pore complex (NPC) via kinesin, and kinesin-mediated transport towards the cell periphery disrupts the NPC and fully disassembles the viral capsid, revealing the viral genome and facilitating its import into the nucleus. It is a widely-held belief that functional adenovirus docking at the nuclear pore complex (NPC) to deliver its genome has already penetrated into the cytosol, and thus is free of endosomal fragments [288, 289, 353, 354].

Using U2OS cells stably expressing mCherry-tagged gal3, we performed live-cell imaging to observe Ad virion trafficking in the cell during entry. We found that viral particles associate with gal3 in the periphery, and translocate to the nucleus while associated with the gal3-positive membranes. Interestingly, in contradiction to previous assumptions, we observed viral egress from ruptured endosomes occurring at the nucleus (Figure 13) [324]. Escape occurs in a fast, directed movement away from the nuclear periphery, indicative of microtubule transport. These data, combined with the reported data suggesting that pIX interacts with kinesin, led us to hypothesize that Ad egress from endosomes away from the nucleus is mediated by pIX associations with kinesin, such that newly cytosolic virus can traffic back to the nucleus and dock at the NPC.



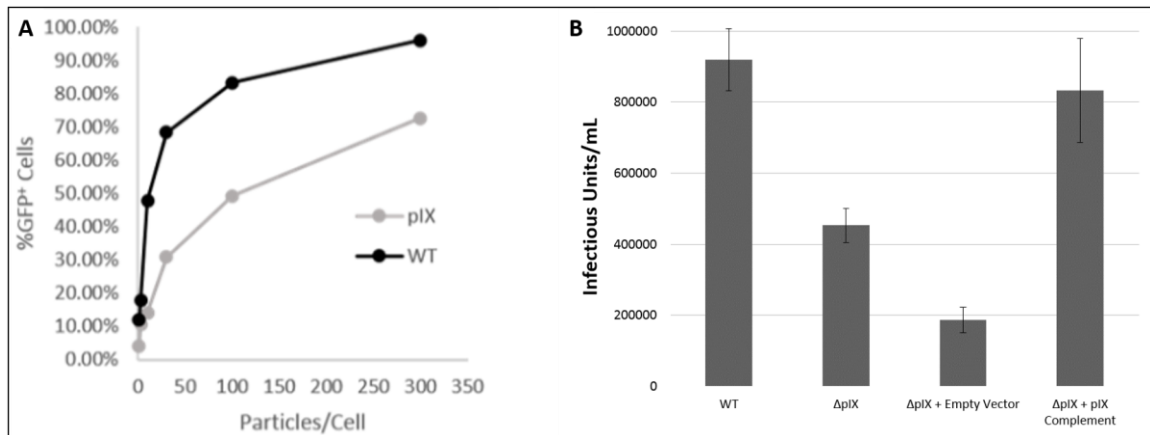
**Figure 13: Time-resolved imaging of Ad5 escape from endosomes.** U2OS cells expressing mCherry-tagged galectin-3 were synchronously transduced with 488-labeled Ad5. Image collection started 30 minutes after transduction, and images were acquired at a rate of 5 frames per second. Particle tracking was performed with MTrackJ.

*Protein IX-deleted Ad is defective for viral infection*

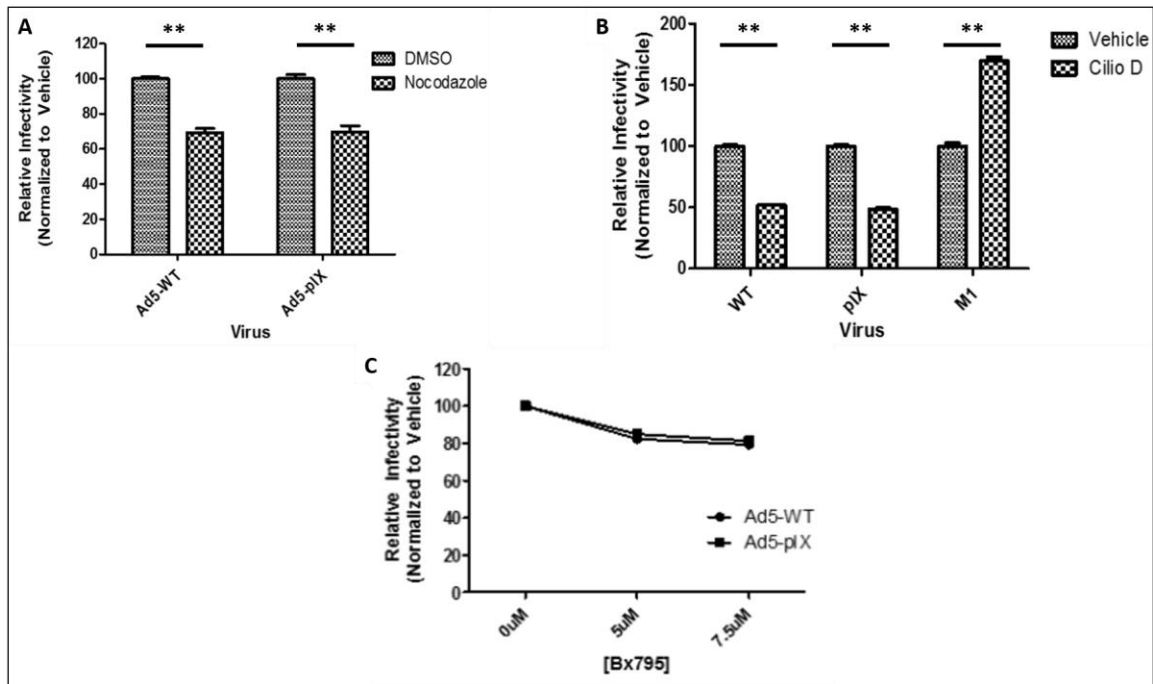
To test our hypothesis, we first generated and characterized a mutant Ad5 virus deleted for pIX (Ad5- $\Delta$ pIX). Previous reports show that Ad5- $\Delta$ pIX exhibits a 2-fold defect in infectivity when compared to WT virus, but where this defect occurs has not been entirely addressed [291]. Initially, it was suggested that pIX is important for genome delivery once the virus docks at the nuclear pore, however preliminary data in our lab may support additional roles of pIX, upstream of nuclear docking during the entry process. We generated our own GFP-expressing Ad virus that contains a 318-bp 5' truncation of the pIX sequence (Ad5- $\Delta$ pIXgfp). This truncation produces an out-of-frame start codon that may potentially generate a 17-amino acid peptide, but this has not been assessed. We assessed the specific infectivity of purified virus, finding a 2-3-fold defect, consistent with previous findings (Figure 14A). Additionally, transfecting pIX on a plasmid into replication-competent virus producer cells restores the defect of Ad5- $\Delta$ pIX compared to empty vector transfections, suggesting the phenotype is specifically due to deleting (Figure 14B).

We attempted to assess where the Ad5- $\Delta$ pIX defect occurs during entry. Indications that pIX interacts with kinesin suggested to us that pIX may act later in entry. To test this, we treated cells with either nocodazole or CilioD to respectively depolymerize microtubules or inhibit dynein and found that, when normalized to vehicle control treated transductions, both inhibitors restrict Ad5- $\Delta$ pIX virus by the same percentage when compared to Ad5-WT (Figure 15A & B). Furthermore, treating cells with Bx795 to inhibit autophagy-mediated restriction has no effect on Ad5- $\Delta$ pIX

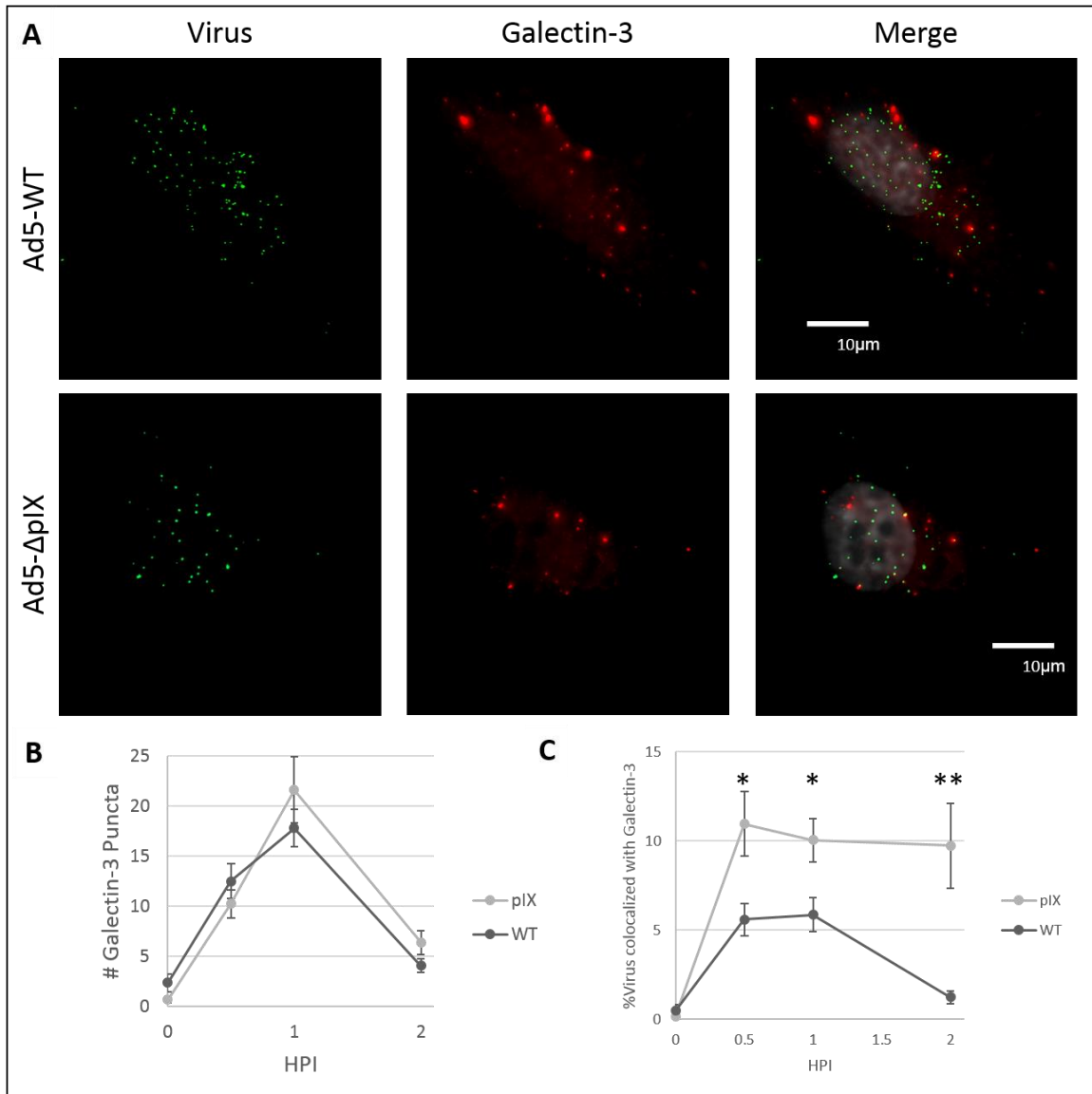




**Figure 14: Characterization of Ad5- $\Delta$ pIX.** **A.** HeLa cells were transduced with Ad5-WT or Ad5- $\Delta$ pIX viruses in increasing physical viral particles per cell. Percent infectivity analyzed by flow cytometry. **B.** 293 $\beta$ 5 cells were transfected with pcDNA 3.1 empty vector or containing full-length pIX, then transduced with Ad5-WT or Ad5- $\Delta$ pIX. 48 hours post-transduction, supernatants were collected and added to HeLas, and GFP transgene expression assessed by flow cytometry.



**Figure 15: Consequences of dynein and TBK1 inhibition on Ad5-ΔpIX infectivity.** A-C. HeLa cells treated with 30μM nocodazole (A), 50μM CilioD (B), or Bx795 (C) were transduced with Ad5-WT or Ad5-ΔpIX. GFP transgene expression was assessed. Ad5-M1 used in CilioD as a control. \*p<0.05, \*\*p<0.001

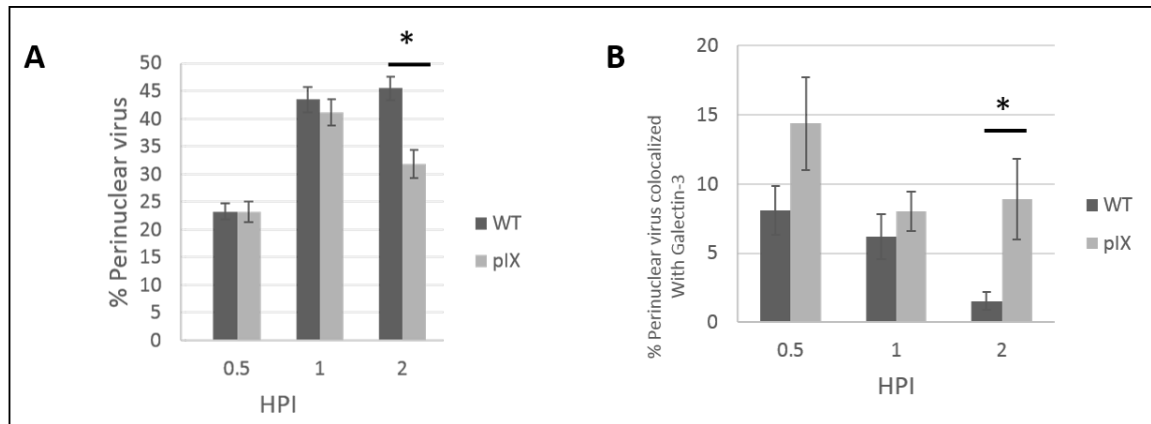


**Figure 16: Influences of pIX-deficient virions on Ad5 entry.** **A.** Representative micrographs of cells transduced with 488-labeled Ad5-WT or Ad5-ΔpIX virus (green). Cells were fixed and stained for galectin-3 (red). **B.** Quantification of galectin-3 puncta present within cells over time. **C.** Percent of virus colocalized with galectin-3. \* $p < 0.05$ , \*\* $p < 0.001$

infectivity, similar to results observed in Ad5-WT transduction (Figure 15C). Taken together, these data indicate that the defect generated by pIX-deleted virions occurs after virion uptake by the cell and its evasion of autophagy.

*Capsid-associated pIX aids virus in endosomal escape*

We next asked whether Ad5- $\Delta$ pIX escapes from endosomes as efficiently as Ad5-WT. We performed synchronous infections with fluorescently labeled virus, and assessed colocalization of virions with gal3 over time. We found that, although both Ad5-WT and Ad5- $\Delta$ pIX viruses induce the same number of gal3 puncta at a given time point (Figure 16B), an increased percentage of Ad5- $\Delta$ pIX virus colocalizes with gal3-positive membranes at 1 and 2 hpi compared to Ad5-WT-transduced cells (Figure 16C). In our dynein inhibition assays we see similar increases in virion colocalization with gal3, so we next asked whether the mutant virus also correspondingly fails to accumulate at the perinuclear area. Interestingly, the number of Ad5- $\Delta$ pIX virions present within 2 $\mu$ m of the nucleus are comparable to Ad5-WT infections at 30 minute and one hour time points, although we do see a small but significant decrease in Ad5- $\Delta$ pIX accumulation at 2 hours post-infection (Figure 17A). However, of those perinuclear virions, again more virus is associated with gal3 in Ad5- $\Delta$ pIX infections compared to WT (Figure 17B), suggesting that virus-containing endosomes traffic towards the nucleus, but the virus cannot escape in a timely manner. Taken together, these data suggest that Ad5- $\Delta$ pIX virus ruptures endosomes as efficiently as the WT virus, but cannot competently egress from them.



**Figure 17: Perinuclear accumulation of Ad5-ΔpIX.** **A.** Percent of Ad5-WT and Ad5-ΔpIX virus targeted to the perinuclear area (within 2μm of the nucleus). **B.** Percent of perinuclear virus colocalized with galectin-3. \*p<0.05

*Cell lines stably expressing pIX enhance Ad infection*

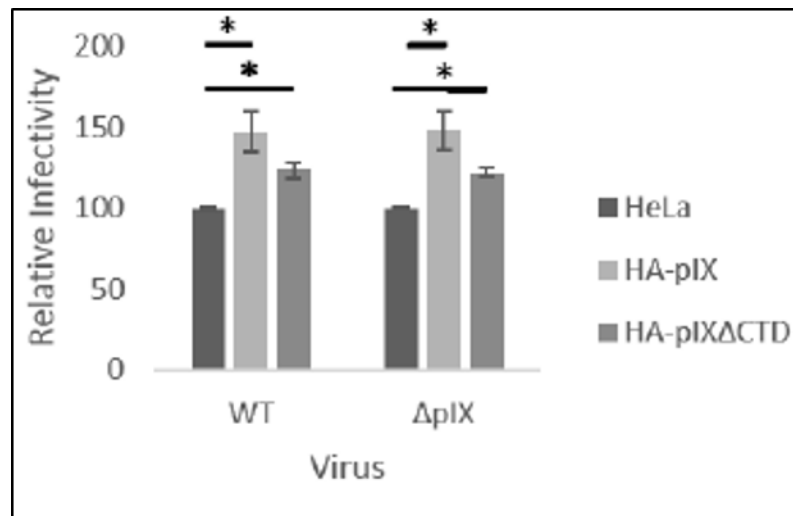
To further elucidate the contributions of pIX during Ad egress from endosomes, we asked what domain of pIX is critical for the WT activity of the protein during virion entry. pIX consists of three putative domains: an N-terminal domain (AAs 1-57) that is important for triskelion formation and integration into the virion capsid, a C-terminal domain (AAs 90-140) that is exposed to the environment upon capsid destabilization, and an alanine-rich linker domain connecting the two. To our knowledge, the function of these three domains in virus-host interactions during the entry steps has not been assessed. Others have postulated that since the C-terminus of pIX becomes exposed upon capsid destabilization, this domain could facilitate interactions with host proteins to aid in entry. We established HeLa cell lines stably overexpressing HA-tagged constructs of either the full-length form of pIX (HA-pIX), or a C-terminal truncated form lacking amino acids 91-140 (HA-pIX $\Delta$ CTD). We hypothesized that if the C-terminal domain is important for virus-host interactions, then the HA-pIX construct will constitutively interact with its binding partner(s), essentially competing for Ad5-WT interactions during virus entry. Conversely, if the pIX CTD is important for such interactions, then HA-pIX $\Delta$ CTD-expressing cells should not actively compete for virus binding, thus HA-pIX cells will restrict Ad5-WT infection while HA-pIX $\Delta$ CTD will not. We also expected that neither cell line would restrict Ad5- $\Delta$ pIX virus. However, we found that compared to the virus transductions in WT HeLa cultures, both virus' infectivities are enhanced almost 50% upon transduction into cells overexpressing HA-pIX (Figure 18). HA-pIX $\Delta$ CTD stable cells also slightly enhance viral infectivity in a pIX-independent manner, but not as

drastically as HA-pIX cells (Figure 18). This result suggests that soluble pIX in cells augments viral infectivity, however the mechanism by which this occurs was not tested.

*Inhibiting KLC1 restricts Ad infection in a pIX-independent manner*

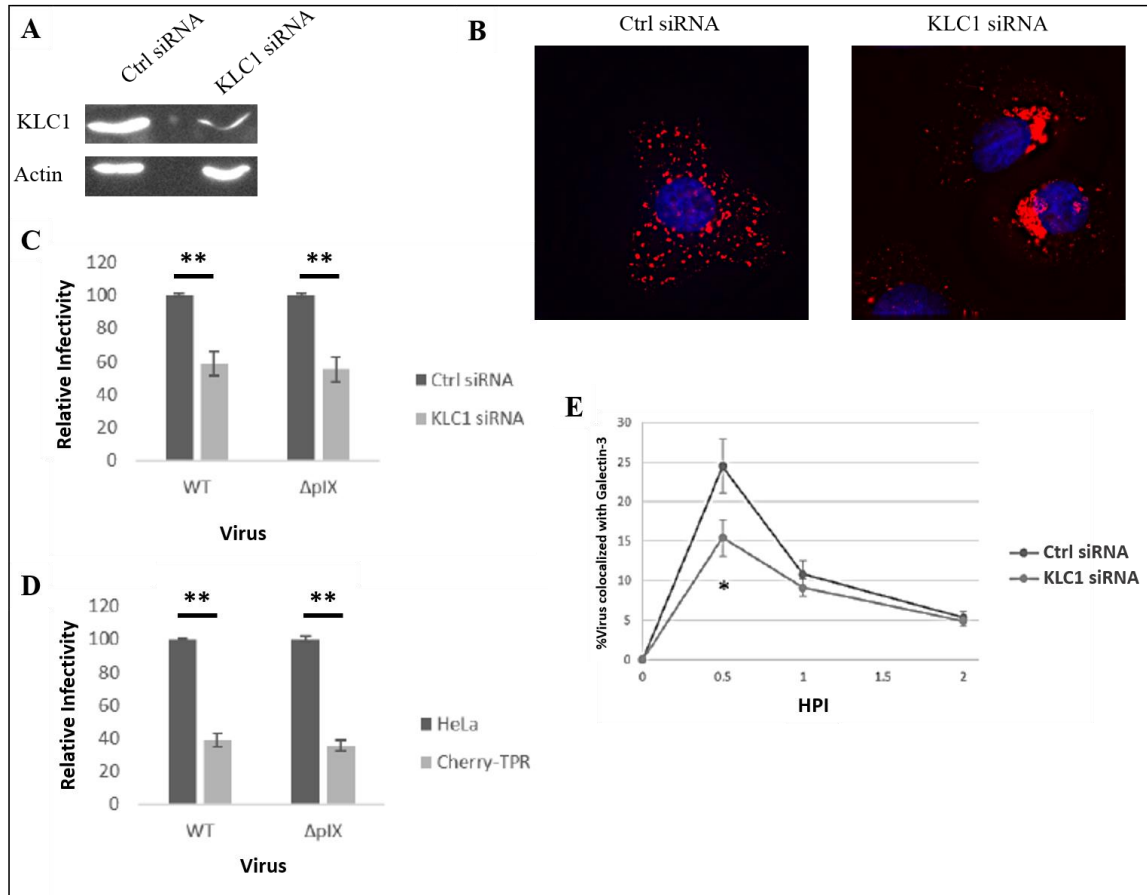
Finally, we sought to address the contributions of kinesin motors in Ad entry. To assess Ad infectivity in the absence of kinesin, we used siRNA to knock down kinesin light chain 1 (KLC1), the component of the kinesin complex responsible for binding cargo [355]. siRNA transfection yielded greater than 70% knockdown of KLC1, and transducing Ad5-WT in knockdown cells significantly decreases kinesin expression, resulting in over 50% restriction in infectivity compared to control siRNA cells, similar to previously published results [291] (Figures 19A-C). As stated above, previous reports propose that kinesin interactions with pIX are important for viral genome delivery. However, whether knocking down KLC1 has an effect on Ad5- $\Delta$ pIX infectivity was not assessed. We found that KLC1 knockdown restricts Ad5- $\Delta$ pIX infectivity just as much as Ad5-WT infectivity when compared to control siRNA cells (Figure 19C). This was striking to us, as it contradicted the implications from previous reports. Instead, these data suggest that pIX is not critical for the contributions of kinesin in Ad infection.

KLC1 knockdown by siRNA may contribute to undesirable effects on cell processes, which could potentially affect general Ad infection, and mask its contributions to Ad entry. To confirm whether the defect in Ad5- $\Delta$ pIX infectivity is due to an inability to interact with kinesin during entry, we generated stable HeLa cell lines overexpressing the pIX-interacting component of KLC1, the tetratricopeptide repeat (TPR) domain. TPR domains are protein-protein interaction domains containing up to six tandem repeats that



**Figure 18: Consequences of pIX overexpression on Ad5 infectivity.** HeLa cells stably expressing HA-tagged full-length pIX or pIXΔCTD were transduced with Ad5-WT or Ad5-ΔpIX virus. GFP transgene expression was assessed by flow cytometry. Relative infectivity standardized to WT cells. \* $p < 0.001$





**Figure 19: Effects of KLC1 knockdown on Ad5 entry.** **A.** HeLa cells were transfected twice with KLC1 or control siRNA. Lysates were probed for KLC1 and actin to assess knock down efficiency. **B.** Cells transfected with control or KLC1 siRNA were fixed and stained for lysosomes with anti-LAMP-1 antibody (red). Control siRNA cells show lysosomal dispersion throughout the cytoplasm (left panel), while cells knocked down for KLC1 accumulate lysosomes at the perinuclear area (right panel). **C.** KLC1 and control siRNA-treated cells were transduced with Ad5-WT or Ad5- $\Delta pIX$  virus. Infectivity assessed by GFP expression is normalized to control siRNA transductions. **D.** HeLa cells stably expressing cherry-tagged KLC1-TPR (Cherry-TPR) were transduced with Ad5-WT or Ad5- $\Delta pIX$  virus, and GFP transgene expression measured by flow cytometry. Infectivity is normalized relative to untransduced HeLa cells. **E.** Colocalization of virus with galectin-3 in KLC1 knockdown cells compared to control siRNA during time course. \* $p < 0.05$ , \*\* $p < 0.001$

are 34 amino acids long [111]. TPRs are responsible for recruiting cargo to the kinesin complex, and ATPase activity within the kinesin heavy chain (KHC) allows the cargo to traffic along microtubules to a specific destination [356, 357]. Our stable HeLa cell lines overexpressing just the TPR domain of KLC1 should act as a dominant-negative form of kinesin: while the TPR can interact with and bind cargo, it cannot form a complex with the KHC, and as such is unable to traffic within the cells by microtubules. We hypothesized that if pIX-TPR interactions are important for infection, then soluble TPR would interact with WT virus, preventing the virus from properly associating with the full kinesin complex, and restrict virus infectivity. Further, if the defect in Ad5- $\Delta$ pIX infectivity is due to an inability to bind kinesin, then there should be no effect on the infectivity of the Ad5- $\Delta$ pIX virus. However, our data confirm the results produced by siRNA knockdown of KLC1, yielding similar restrictions in both virus' infectivity (Figure 19D). Taken together, these data suggest that although the defect in Ad5- $\Delta$ pIX occurs prior to endosomal escape, it is not due to an inability of the virus to interact with kinesin.

#### *KLC1 knock down enhances early Ad escape from endosomes*

Although KLC1 knock down restricts Ad infectivity in a pIX-independent manner, we were still interested in further characterizing the contributions of kinesin in Ad entry. We reasoned that since our live cell imaging assays show viral egress from endosomes towards the cell periphery, kinesin knock down may limit Ad egress from ruptured endosomes. To test if KLC1 knockdown affects endosomal escape, we used IFA to assess Ad5-WT virus colocalization with gal3 in KLC1 or control siRNA-transfected

cells. We found no difference in the ability of virus to rupture endosomes as assessed by the number of gal3 puncta, nor in the percent of virions colocalized at the nucleus.

However, although we found similar percentages of virions contained within ruptured endosomes at later times post-entry, we reproducibly saw that virions in KLC knocked down cells colocalize with fewer gal3 puncta at the 30 minute time point compared to control cells (Figure 19E). Since the number of ruptured endosomes is consistent, these results suggest that, opposite to our hypothesis, kinesin actually confines Ad to ruptured endosomes early in the entry process. Furthermore, as knocking down kinesin elicits no difference in WT virus to egress from ruptured endosomes at later times post-entry, this suggests that kinesin's contribution to infection may be early in the entry process.

## **CHAPTER IV**

### **DISCUSSION**

Viruses have co-evolved alongside their hosts for millions of years in a sort of arms race, with the host continually developing mechanisms to restrict viral infection, and vice versa the virus improving its methods to elude detection and subsequent restriction. Viruses have the capacity to specifically manipulate cell activities to their advantage, making them great tools in probing very complex pathways and protein functions within the host. Elucidating both the mechanisms of host restriction of viruses, as well as methods of pathogen evasion of such restrictions, are important to design treatments for preventing disease. Our lab uses adenovirus (Ad) to probe some of the host responses to pathogen infection and assess the approaches utilized by Ad to elude restriction. Understanding the contributions of distinct mechanisms of evasion that allow for optimal Ad genome delivery are also useful in the context of replication-defective Ad vectors, which are currently being pursued as potential vaccine candidates to deliver transgenes to the nucleus. As such, this study focuses on host responses to Ad as a barrier to viral entry and genome delivery. We specifically concentrate on mechanisms of viral evasion of restriction by autophagy after endosomal rupture and the methods of viral egress into the cytoplasm.

## Consequences of Ad-induced membrane rupture

### *Significance of galectin recruitment*

Our lab and others showed that galectins 3, 8, and 9 accumulate at endosomes ruptured by entering pathogens [72]. Knocking down galectin-8 in cells enhances *Salmonella* replication, however gal3 and gal9 knock down experiments showed no effect on replication. Similarly, we previously showed that siRNA against gal8 restores Ad5-M1 infectivity, suggesting that gal8 is important for host restriction of Ad5 in a pVI-PPxY-dependent manner. Gal8 recruits NDP52 to sites of damage faster than the ubiquitin-binding process in *Salmonella* infections (1 hour compared to 4 hours, respectively). This is especially important in Ad infection, as most virus would already have the opportunity to deliver its viral DNA to the nucleus by 4 hours. This result implies that gal8 may have a specific role during pathogen-induced autophagy rather than the autophagic process required for general host cell maintenance.

It is interesting that gal8 knockdown enhances pathogenicity but there is no obvious change when similar knock downs are performed for gal3 and gal9. Both gal8 and gal9 contain tandem-carbohydrate recognition domains connected by a linker sequence. Gal8's C-terminal carbohydrate recognizing domain (C-CRD) has a higher affinity for N-linked glycans than its N-terminal CRD (N-CRD), suggesting that the C-CRD binds cell surface glycans revealed after endosomal rupture [358]. Gal8 and gal9 C-CRDs have 60% similarity, implicating that they both have the capacity to bind these sugars. However, although gal8 and gal9's N-CRDs share ~53% similarity, they each interact with different saccharide molecules [358-360]. Additionally, while gal8 is widely

distributed across most tissues, gal9 is primarily found in the kidney, thymus, and synovial fluid. These differences may account for both galectins having the capacity to bind ruptured endosomes, but only gal8 enacting a restrictive effect on incoming pathogens.

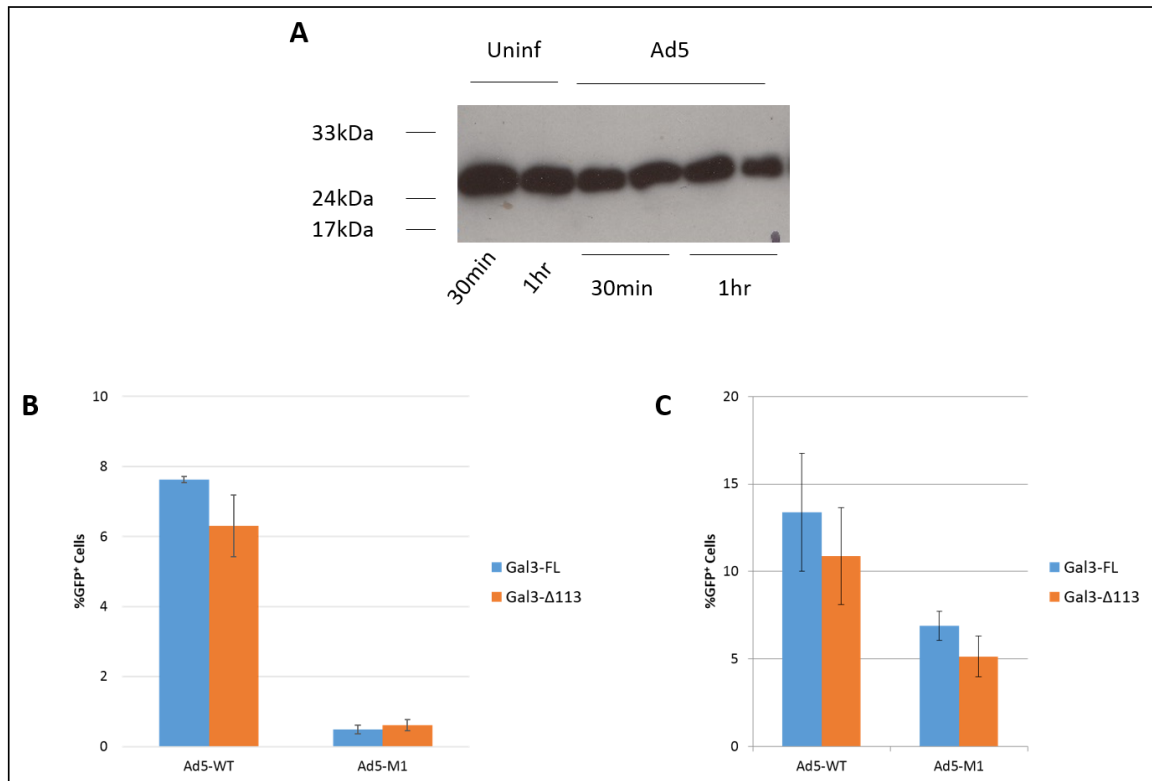
Galectin-3 is structurally unique in that it contains one CRD at its C-terminus, connected to a disordered N-terminal tail containing 8 tandem repeats of the sequence Y-P-G-X(3)-P-G-A. In high concentrations, gal3 oligomerizes into pentameric structures through this tail domain, and although N-terminally-truncated mutants still bind sugar moieties, the tail is essential for extracellular gal3 signal transduction and cell adhesion functions [361-365]. We initially hypothesized that endosome-associated gal3 oligomerizes via its tail to cage Ad virions from efficiently egressing. Precedence for this proposal arose from noticing that the gal3 tail sequence contains a LXGG sequence motif downstream of the N-terminal tail, at the beginning of the CRD. Ad protease (AVP) recognizes and cleaves LXGG sequences, which led us to ask whether virion-associated AVP has the capacity to cleave gal3 during entry. Additionally, previous reports show that during *Leishmania donovani* (*L. donovani*) infection, extracellular gal3 binds bacterial glycans to act as a DAMP upon encountering macrophages. However *L. major* cleaves the tail domain of gal3, restricting its function [366]. These data led us to assess whether Ad protease cleaves gal3 and test if a 113-amino acid N-terminally truncated gal3 acts as a dominant negative protein to enhance Ad infection. However, we found that Ad transduction does not cleave gal3 within one hour after entry, and stably expressing a cherry-tagged N-terminal truncation of gal3 in MEFs (which do not

endogenously express gal3) or HeLa cells has no effect on Ad5-WT or Ad5-M1 infectivities compared to full-length gal3 (Figure 20).

These results, in addition to the results reported in *Salmonella* replication upon gal3 knockdown, indicate that although gal3 and gal9 are recruited to sites of rupture, they have no function with regard to intracellular infection *in vitro*. All three of these galectins function in extracellular signaling pathways, and must be secreted from the cytoplasm (reviewed in [367]). Perhaps they are simply present in the cytoplasm prior to secretion, and encountering glycans in the cytosol keeps them within the cell. Intriguingly, galectins do not contain classic secretion signal peptides [368]. It is possible that galectin secretion relies on exocytosis mediated by the autolysosomal pathway, and galectin-bound endosomal fragments act as a danger signal to neighboring cells. However, as neither of these proposals have been examined this remains pure conjecture.

#### *TBK1 contributions to autophagosome formation*

Ads must escape from endosomes to properly traffic to the nucleus. To do so, the virus fragments the endosome by utilizing the membrane lytic activity of its capsid protein VI (pVI). We show here and in other reports that membrane rupture alerts the cell to infection, activating a number of defenses. This response mediates both innate and adaptive responses in a cell, allowing a healthy host to limit infection. By using a direct marker of adenovirus-mediated vesicle rupture, we can elucidate the innate responses in a temporal manner, and further understand how the virus attempts to inhibit the virus intrinsically before it can establish an infection.



**Figure 20: Ad5 infectivity in cells expressing N-terminal tail-deleted galectin-3. A.** HeLa cell lysates transduced with Ad5-WT were probed for galectin-3 to assess cleavage. **B-C.** HeLa cells (**B**) or MEFs (**C**) stably expressing mCherry-tagged full-length (Gal3-FL) or a 113-amino acid N-terminally-deleted galectin-3 (Gal3-Δ113) were transduced with Ad5-WT or Ad5-M1. GFP-positive cells were assessed by flow cytometry.



Autophagosome formation around damaged endosomes is a near-immediate response to vesicle rupture, and one of the first attempts for the cell to restrict viral penetration into the cytosol. Mutant Ad virions unable to efficiently egress from ruptured endosomes before sequestration by autophagy are delivered to lysosomes for degradation. Our lab and others suggest the host's quick response to pathogen-mediated vesical damage is facilitated by gal8-dependent recruitment of restrictive molecules [72]. In *Salmonella* infections, gal8 recruitment of NDP52 is necessary to mediate restriction. However, we previously found that knocking down NDP52 restricts Ad5-WT infectivity but has no effect on Ad5-M1 [324]. This result suggests that, in contrast to the data reported in the *Salmonella* model, gal8 recruitment of NDP52 is important for optimal Ad infection rather than viral restriction. Perhaps Ad infection differentially triggers gal8 interactions with NDP52 compared to *Salmonella*-mediated pathogenesis. How this occurs and its relevance in signaling pathogen evasion may reveal subtle but distinct mechanisms utilized by the host to specifically target unique pathogens for restriction.

Our data also suggest that the adaptor protein kinase TBK1 is essential for autophagy machinery to sequester the endosome-associated Ad virions. These data strongly implicate a role for one of the adaptor molecules, p62 or optineurin (OPTN), in resolving Ad restriction by the host. Similar to NDP52, preliminary data do not implicate p62 in Ad restriction (data not shown), leaving OPTN. Whether OPTN is recruited to membrane damaged by Ad has not been assessed. IFA experiments to test OPTN recruitment to ruptured endosomes and transduction assays in OPTN knock down cells will begin to elucidate the contributions of this adaptor, if any. Further investigations

assessing whether Ad entry induces OPTN phosphorylation in a TBK1-dependent manner may further resolve the mechanisms of autophagosome formation during Ad infection.

We also show here that that treating HeLas with Bx795 prevents Ad-dependent LC3 cleavage after 1 hour synchronous infection. This differs from previous reports that LC3 cleavage occurs in *tbk1*<sup>-/-</sup> MEFs or MEFs treated with Bx795, but they do not mature and fuse with lysosomes [83]. This result may be due to cell type differences (cultured vs. primary cells), or a difference in the way autophagy is induced in either system. In our system, autophagosome formation is induced by vesicle damage, whereas in the MEF assays, cells were starved for 4 hours to facilitate autophagy. Starvation-mediated autophagy occurs via mTOR inactivation, which our data suggest does not regulate Ad-mediated autophagy. Although p62 phosphorylation by TBK1 is critical for autophagosomal maturation in the MEF assays, these observations may suggest that TBK1 functions differently depending on the mechanism of signaling. Further studies are required to elucidate TBK1 activities upon signaling through different pathways.

Previous reports implicate that host sensing of Ad through the cGAS/STING complex stimulates TBK1 to promote IRF3 activity [369-371]. Inhibiting signaling via this complex in both cell lines and primary cells reduces mRNA transcription of molecules important for type I IFN responses, including IFNB, STAT1, and ISG15. Our data, in conjunction with these studies, suggest that TBK1 is important to properly mediate antiviral activity against Ad, both as a signaling component for mounting a suitable innate immune response and as a restriction element during autophagosome

formation. Our observations contribute to the growing body of literature that implicates TBK1 as a critical node or junction kinase, similar to mTOR and PKA, which function in sensing distinct viral DAMPS and accurately directing signals through explicit host signaling pathways to mediate specific antiviral responses (reviewed in [372]).

One further caveat to our TBK1 studies is that inhibition via Bx795 treatment is also reported to inhibit five other host kinases: IKK $\epsilon$ , PDK1, Aurora B, ERK8, and MARK3 [327]. Although Bx795 inhibits these kinases >5-fold less potently than TBK1 at micromolar concentrations, it is worth considering whether affecting their activities may skew our results. PDK1 and Aurora B inhibition would stimulate autophagy through mTOR inactivation; since mTOR function is dispensable for Ad-mediated autophagy induction, these are likely not involved in restricting Ad. Furthermore, we do not see a difference in the number of LC3 puncta induced in cells treated with Bx795 or vehicle control, indicating that these kinases are not affected. MARK3 is a microtubule-associated kinase, and is not implicated in autophagy. IKK $\epsilon$  interacts with TBK1 to activate NF- $\kappa$ B [373], but no literature reports an involvement in autophagosome maturation. However, observations do implicate autophagy stimulation through ERK8 [374]. ERK8, or MAPK15, localize to starvation-induced autophagic structures via LC3 interactions, and its activation is mediated by hydrogen peroxide-induced ROS production. Thus, Bx795 treatment may implicate a role for ERK8 in Ad infection in addition to, or instead of, TBK1. To further address this, Ad transduction studies can be performed using either other chemical inhibitors of TBK1 or by stably overexpressing a kinase-dead mutant form of the protein. Chemical inhibitors may be preferential since

they are typically reversible, minimizing potential long-lasting effects from dominant negative expression assays. One inhibitor, MRT67307, might be useful in this application [375]. Nanomolar concentrations of this drug inhibit nearly 90% of TBK1 activity, but less than 20% of ERK8 activity. Additionally, with the exception of MARK3, it does not inhibit the other kinases affected by Bx795. This inhibitor would be useful to specifically demarcate the contributions of TBK1 to autophagy induction.

*ROS influences Ad-induced autophagy signaling*

The autophagy-inducing signaling molecules activated to restrict Ad during entry in a pVI-PPxY-dependent manner still remain unknown. Many bacteria that rupture endosomes activate autophagy via an mTOR-dependent pathway, mimicking amino acid starvation in the infected cell [58-60]. Data from our collaborator suggest that mTOR signaling does not affect Ad-mediated restriction by autophagy, implying that membrane damage is somehow differentially recognized in cells depending on the mechanism of rupture. We corroborated this evidence using inhibitors of the AMPK pathway, which is an upstream signaling molecule of mTOR. Although gal8 knockdown enhances *Salmonella* and Ad infections, autophagy is still activated in these cells but not found colocalized with pathogens, indicating that gal8 is involved in properly targeting autophagic machinery rather than inducing it. Clarifying whether there are differences in host cells recognizing damaged membranes will further aid our understanding how different pathogens mediate and exploit host responses and processes.

We have seen previously that high concentrations of ROS are quickly produced upon membrane rupture by Ad, leading to the logical proposal that ROS is involved in

such signaling. However, we find here that multiple ROS-mediated signaling pathways have no differential effects on Ad5-WT or Ad5-M1 infectivities. Inhibiting either ERK1/2 or p38 has no effect on Ad infectivity, similar to the results found upon inhibiting mTOR signaling pathways. Additionally, preliminary data suggest that treating cells with the ROS scavenger N-acetyl-cysteine restricts both viruses similarly (data not shown). These data implicate a pVI-PPxY-independent importance of ROS during infection and further suggest that ROS does not facilitate autophagy-mediated restriction of Ad5-M1, and likely do not merit continued investigation in regards to PPxY-dependent autophagy evasion. Further studies are required to pinpoint the signaling pathway(s) induced by Ad membrane damage to initiate autophagosome formation.

*Beclin-1 and the PI3K signaling pathway in Ad-induced autophagy*

We previously showed that knocking down beclin-1 by siRNA restores Ad5-M1 infectivity 2-fold, indicating a role for beclin-1 in signaling to activate autophagosome formation. Beclin-1 is a major component of mTOR-dependent and -independent autophagosome initiation. Beclin-1-mediated autophagy requires a core complex consisting of Beclin-1, a protein kinase Vps15, and the class III phosphoinositide 3-kinase (PI3K) Vps34. In mammalian cells, this core complex interacts with over 10 different regulatory proteins to mediate autophagosome formation and/or vesicle trafficking (reviewed in [376]). Unsurprisingly, another pathway seemingly involved in autophagy-mediated restriction of Ad is PI3K signaling. We previously showed that treating cells with the class I/III PI3K inhibitor 3-MA also partially restores Ad5-M1 infectivity approximately 2-fold. Interestingly, treating beclin-1 knockdown cells with 3-

MA synergistically enhances Ad5-M1 infectivity 8-fold, but only improves Ad5-WT activity by 30%. These data suggest that PI3K and Beclin-1 act epistatically to restrict Ad entry, and the pVI-PPxY motif is critical to evade this restriction. However, another publication proposed that 3-MA has a dual role in autophagic flux depending on the cell conditions [377]. Their results show that while treating cells with 3-MA does restrict autophagy induction under starvation conditions, it surprisingly enacts the opposite function when cells are under normal “full” conditions, that is cells fed DMEM supplemented with 10% FBS. We have previously shown that exhausting autophagy machinery via transfecting cells with an empty plasmid vector fully restores Ad5-M1 infectivity. Thus, under our conditions, treating cells with 3-MA prior to Ad5-M1 transduction may partially exhaust autophagy machinery, enhancing infectivity. The synergistically enhanced phenotype observed in beclin-1 knockdown cells may be due to further inhibiting the capacity of autophagosome nucleation.

Designing experiments to reveal autophagy induction by Ad can be difficult, as many previously established experimental systems that limit autophagy do so by temporally changing autophagic flux instead of shutting down its actual stimulation. For adenovirus, these results can skew our interpretation of the data. As such, experiments need to be carefully designed to specifically assess mechanisms of autophagy induction rather than changes in autophagic flux. 3-MA enhancement of autophagy under nutrient-rich conditions starts between 3 and 6 hours of treatment, which could suggest that 3-MA's effects on autophagy would not affect Ad5-M1 infectivity, but more studies need to be performed to fully tease out this pathway. Wu, et al. further show that while 3-

MA's effect on class I PI3K activity is long-lasting, its inhibitory effects on class III activity is transient. The class III PI3K complex interacts with beclin-1, adding more complexity in interpreting these results. In contrast, their data suggest that another PI3K inhibitor, wortmannin, transiently affects class I PI3K, but persistently inhibits class III PI3K, even after 9 hours. It would be interesting to determine if the same enhancement in Ad5-M1 infectivity is seen in beclin-1 knockdown cells treated with wortmannin compared to 3-MA. This experiment would strengthen the results observed with 3-MA, further implicating PI3K involvement in Ad5-M1 restriction.

If we maintain that both PI3K signaling and beclin-1 are critical in nucleating autophagosome formation, it is logical to assess what proteins are in complex with these molecules during Ad entry. Bcl-2 is a negative regulator of beclin-1, which perpetually binds beclin-1 unless physically displaced by competing protein-protein interactions or post-translational modifications (PTMs) on either protein. Bcl-2 must be displaced to activate beclin-1 and induce autophagy. Our data here suggest that chemically blocking Bcl-2 phosphorylation by JNK or ERK in the host has no effect on Ad infectivity, suggesting that Bcl-2-specific PTMs do not mediate autophagosome formation during Ad entry. However, these data do not eliminate the possibilities that Bcl-2 is supplanted by another protein or if beclin-1 is altered by PTMs. Testing whether Bcl-2 displacement or PTMs are important for beclin-1-mediated Ad restriction would further help to determine the specific mechanisms of Ad-induced autophagy. Previous students in our lab tested the effects of Bcl-2 overexpression on Ad in THP-1 cells. We expected that if Bcl-2 competitively interacts with beclin-1, then overexpressing Bcl-2 will restore Ad5-M1

infectivity but have no effect on Ad5-WT. However, their experiments found no effect on Ad5-M1 infectivity (data not shown). This result suggests that increasing Bcl-2 concentration in the cell, and thus its capacity to bind beclin-1, is not important for mediating Ad infection. Alternatively, these data may suggest that beclin-1 is post-translationally modified to remove Bcl-2 during Ad entry, potentially explaining why increasing Bcl-2 in the cell has no effect on Ad infectivity.

Additional tests can be performed to assess if beclin-1 is altered by PTMs during Ad transduction, such as assessing its ubiquitination or phosphorylation status. If PTM modification does occur, we can further focus on assessing the proteins involved in Ad-induced autophagosome formation. Reports indicate that beclin-1 is ubiquitinated by TRAF6 on Lysine-117 or phosphorylated by DAPK on Threonine-119 [378, 379]. To date, neither TRAF6 nor DAPK are implicated in Ad entry. If beclin-1 is modified by these proteins, then we can test whether ubiquitination or phosphorylation is a critical factor. An easy assessment of Beclin-1 PTM is to probe for beclin-1 on western blot during early Ad transduction. If Beclin-1 is ubiquitinated, then a clear shift in molecular weight from ~50kDa to 58kDa or higher should be observed. Alternatively, phosphorylation can be assessed using Phos-Tag SDS-PAGE, where phosphorylated proteins run through the gel much slower than non-phosphorylated proteins. Therefore, comparing infected lysates to uninfected lysates would inform us which PTM occurs upon Ad entry. Testing whether either TRAF6 or DAPK is specifically important in Ad entry could be performed via chemical inhibition or siRNA knock down of either protein. TRAF6 activity is important in NF- $\kappa$ B responses during Ad infection, and is activated



through TLR4 signaling, but this occurs late in infection [380]. DAPK has no known effect on Ad infection. It is regulated by calmodulin and increases caspase-mediated cell death in HHUA epithelial cells [381]. Thus, knocking down DAPK may not be a viable experimental system and may require a more intricate assessment. Rather than knocking down these proteins, we could instead generate cell lines expressing beclin-1 alanine point mutants which cannot be either ubiquitinated or phosphorylated. We would expect that if either PTM is important for Ad5-M1 restriction, then cells containing point mutants would show enhanced Ad5-M1 infectivity.

If the Bcl-2-beclin-1 complex is dissociated through protein competition, then we can assess the contributions of other proteins that regulate beclin-1-mediated autophagy induction. Many molecules are implicated in such regulation, including UVRAG and Atg14L [382-384]. These regulators are both implicated in activating autophagy through beclin-1, however they bind beclin-1 mutually exclusively of each other [385]. UVRAG knock down in cells does not affect autophagic flux, but mainly localizes to Rab9-positive late endosomes. Atg14L is found in the ER and associates with membranes containing positive curvature [386]. It is implicated in both autophagosomal initiation and LC3 conjugation to phagophore membranes [383, 384]. It recruits class III PI3K to the ER and partially colocalizes with the autophagy initiation molecule Atg16L. This may implicate a role for Atg14L-mediated autophagy during Ad entry. Atg14L-induced autophagy is hijacked by the intracellular pathogen *Anaplasma phagosytophilum*, presumably to deliver nutrients to the bacterium [387]. However, whether Atg14L is important in viral infections is not well characterized. Testing whether UVRAG or

Atg14L is important for Ad5-M1 restriction can further delineate Ad-mediated induction of autophagy.

Taken together, these data suggest that Ad-mediated membrane rupture during entry induces autophagosome formation through mTOR-, ROS-, and starvation-independent mechanisms. Beclin-1 and the class III PI3K complex appear to be intimately involved in restricting Ad5-M1, suggesting that endosome rupture stimulates anti-viral innate responses through this complex. Performing the assays proposed above will help delineate the specific contributions of beclin-1-associated host molecules in inducing autophagosome formation to restrict Ad entry. Other studies to determine additional host proteins recruited to sites of membrane damage by Ad may also direct future investigations in elucidating mechanisms of Ad escape. Overall, this will not only help our understanding of Ad evasion of autophagy, but also may contribute to our comprehension of the complexity of autophagy signaling pathways and how various stimuli differentially induce autophagosome formation.

#### **Protein VI PPxY motif – recruiting host proteins**

Data from our lab clearly indicate that the pVI-PPxY motif is required for viral evasion of autophagy and penetration into the cytoplasm. These results suggest that endosomal escape is important for Ad evasion of host restriction responses, but that does not seem to be the whole story. We see that Ad deleted for pIX (Ad5- $\Delta$ pIX) is also retained in endosomes, but is not restricted by autophagosomes (Figures 15 & 16). Live cell imaging experiments suggest that WT virions traffic through the cell while remaining within gal3-positive membranes, only egressing once they traffic to the nuclear

periphery. These observations suggest that evading autophagy depends on more than just endosomal escape, implicating a multi-step process during viral egress: virus damages endosomes, moves away from sites of targeted autophagy machinery to evade restriction, then egresses from endosomes at a later time once the virus is no longer under threat of sequestration. Perhaps the PPxY motif mediates viral trafficking away from sites of autophagosome formation, and some other protein – possibly pIX – is required to facilitate the subsequent act of penetrating into the cytosol. This hypothesis requires more testing to elucidate the dynamics of Ad entry over time. Specifically, understanding what the PPxY motif recruits and/or interacts with will reveal its importance in evading sequestration by autophagosomes.

Other reports indicate that the pVI-PPxY motif is important to interact with Nedd4-family ubiquitin ligases in the cytoplasm, and is necessary to restrict the PML-associated host restriction factor Daxx [209, 325]. Nedd4.2 ubiquitinates pVI-WT but not pVI-M1, and siRNA against Nedd4.2 partially inhibits Ad5-WT infectivity, but not to Ad5-M1 levels. Similarly, Daxx-depleted cells partially restore Ad5-M1 infectivity, but not completely to WT levels. These reports suggest a 2-pronged requirement for pVI-PPxY during infection: first during entry, Nedd4.2-dependent ubiquitination affects the protein's function; and second, once the viral genome is delivered to the nucleus, pVI inhibits Daxx from interfering with early stages of replication, and further appears important for initial viral transcription. Although the second prong is important, it is necessary to design experiments that separate these effects to specifically assess the contributions of pVI-PPxY during entry.

*The contributions of pVI ubiquitination*

The PPxY-dependent interaction with Nedd4.2 enables Ad accumulation at the nucleus, implicating that this process is important for pVI function during entry. However, the contributions of pVI ubiquitination by Nedd4.2 are still unresolved. Moreover, whether recruited Nedd4.2 ubiquitinates other viral or host proteins, and the influences of these modifications are also unknown. However, it is practical to initially focus on whether pVI ubiquitination contributes to viral entry. Transiently transfected pVI-WT highly associates with microtubules, but pVI-M1 does not.

Whether microtubule interactions are dependent on the ubiquitin status of pVI is unknown, although the observation that Nedd4.2 knockdown reduces Ad accumulation at the nucleus would suggest that this is the case. Intriguingly, the Wodrich lab has mapped pVI ubiquitination to a single lysine. They found that mutating this residue to arginine or alanine has no effect on viral infectivity, suggesting that specific ubiquitination of pVI is dispensable for Ad entry. This result further implies that pVI recruitment of Nedd4.2 is important to mediate the ubiquitination of other proteins, or enacts some other function at the site of vesicle rupture. Whether these proteins are viral, host, or both is not yet identified. Penton base also contains PPxY motifs, and is ubiquitinated when over-expressed in cells. Whether Nedd4.2 is important for this ubiquitination, and if there is a physiological relevance of this ubiquitination in the context of infection, is unexplored. Clearly, Nedd4.2 within the cell is important for efficient Ad infection. These studies will hopefully provide more evidence for its role during the entry process.

*Host protein interactions with pVI*

Pull down experiments performed here suggest that purified pVI interacts with multiple host proteins, but does not clarify whether these are direct interactions or via pVI interacting with protein complexes. Additionally, these are likely interactions that occur with non-ubiquitinated pVI, as the purified protein is incubated with cell lysates, however the ubiquitination status was not tested. It is unlikely that Nedd4.2 proteins have ubiquitination activity in the absence of excess ubiquitin and ATP, and at the low incubation temperatures. The current conditions can be altered to assess whether the pVI ubiquitination status changes the profile of pVI-host protein interactions. Incubating lysates with pVI at physiological temperature in the presence or absence of ATP and purified ubiquitin would allow endogenous Nedd4.2 potentially interacting with pVI to ubiquitinate pVI. The ubiquitination status of pVI would be assessed by immunoblotting for pVI, and we would anticipate multiple pVI-positive bands if ubiquitination is occurring. If pVI ubiquitination affects viral-host protein interactions, then we would expect to see a distinct banding pattern in lysates containing ATP and excess ubiquitin. GFP-lysate samples, as well as ATP- and ubiquitin-free controls would shed light on any differences observed in the pull down assays.

Furthermore, we could not specify which interactions during the pull down assays are pVI-PPxY-specific, as we were unable to purify pVI-M1 protein from bacterial lysates, likely due to its lytic activity. Instead of attempting to purify full-length mature pVI, future studies could alternatively perform these studies using pVI lacking the membrane lytic domain. These studies would purify truncated pVI-WT and pVI-M1 and

perform pull down assays, using mature pVI-WT and GFP proteins as positive and negative controls, respectively. This could also potentially yield cleaner pull downs, as it should not pull out anything bound by the lytic  $\alpha$ -helical domain. We would expect that truncated pVI-M1 would not pull down one or more of the proteins pulled down by pVI-WT. These could then be sent off for identification, and focus attention on the relevant interaction.

## **Microtubules**

### *Contributions of microtubule motors in aiding viral escape*

Microtubules (MTs) are the major network through which many host molecules traffic throughout the cell. Its maintenance is absolutely essential for cell movement, division, and survival. Thus, it is not surprising that many pathogens take advantage of the MT network to mobilize to their target destination. We find here that Ads require both MT motors dynein and kinesin for efficient infection, and both are involved in efficient virion penetration into the cytosol.

#### *Dynein*

A myriad of previous studies focus on the contributions of dynein to adenovirus cell entry. Inhibiting dynein via siRNA knock downs and protein overexpression have clearly implicated its role in entry and proper viral targeting to the nucleus, but these types of assays have severe pleiotropic effects on global cell function and growth. Additionally, the specific point in entry that the virus recruits and interacts with dynein in these assays has not been fully characterized. We utilized a recently developed small molecule inhibitor of the dynein motor function, called ciliobrevin D (CilioD) to

specifically ask at what stage in entry does Ad initially recruit dynein, and what the contributions of the pVI-PPxY motif are in virus-dynein interactions. Using this small molecule inhibitor allowed us to immediately probe the role of dynein in Ad entry (i.e., over the course of hours) without drastically affecting the motor's function over a long period of time (i.e., days), as is the case with siRNA or overexpression models.

We found that treating cells with CilioD restricts Ad5-WT infectivity 60%, which corresponds to increased colocalization with gal3-positive endosomes during entry (Figure 10). Previous dynein inhibition studies suggest that dynein-virus interactions occur after the virus penetrates into the cytosol, and so to our knowledge these are the first data to implicate a defect in viral escape in the absence of efficient dynein motor activity. We further found that Ad5-M1 infectivity is partially restored upon inhibiting dynein (Figure 10). In fact, when transduced with the same physical number of particles per cell, Ad5-WT and Ad5-M1 specific infectivities were similar when treated with 100 $\mu$ M of the inhibitor (data not shown). These results imply that the defect in Ad5-WT observed upon inhibiting dynein is absolutely dependent on the pVI-PPxY motif. The partial restoration we observe during Ad5-M1 transduction is likely to an inability to lysosomes and/or autophagosomes to properly mobilize and fuse together, since other data indicate that lysosome transport throughout the cell requires dynein function.

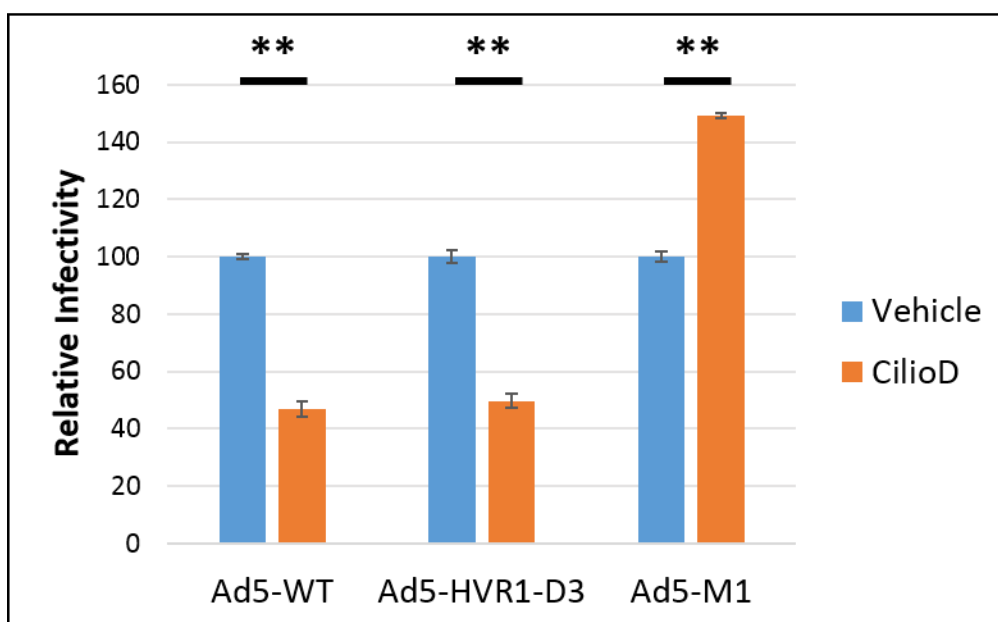
The next logical step to assess in this model is determining how Ad capsid specifically exploits dynein after membrane rupture. Published data suggest that the major capsid protein hexon interacts with dynein to mediate transport to the nucleus [290, 388]. These authors show that pH-primed hexon (pH 4.4) interacts with dynein *in vitro*,

and that this interaction is dependent on the hypervariable region 1 (HVR1) sequence in hexon. Our lab previously generated an Ad mutant that replaced the hexon HVR1 with an epitope from the *Plasmodium falciparum* protein, Pfs25 (Ad5-HVR1D3). This replacement removes the putative HVR1 sequence responsible for dynein interactions.

We found that Ad5-HVR1D3 has a modest ~2-fold defect in infectivity, so with the data from the other reports in mind, it stood to reason that this virus' defect is due to an inability to interact with dynein. To test this, CilioD- or DMSO-treated cells were transduced with Ad5-WT and Ad5-HVR1D3, and relative changes in infectivity were assessed. We predicted that if the Ad5-HVR1D3 defect is due to an inability to interact with dynein, then cells transduced with Ad5-HVR1D3 in the presence of CilioD should have no difference in infectivity compared to DMSO controls, or perhaps a partial increase in activity similar to that observed with Ad5-M1. However, we found that treating cells with CilioD restricted the Ad5-HVR1D3 virus' relative infectivity just as much as Ad5-WT-transduced cells (Figure 21). These results indicate that, in our hands, hexon HVR1 is dispensable for Ad5 utilization of dynein. Additionally, as its name suggests, Ad5 HVR1 is hypervariable and is not conserved among other Ad species. Thus, it is difficult to believe that a poorly conserved protein sequence from a single Ad species is responsible for the virion's critical interaction with dynein.

The results from the Vallee lab with pH-primed hexon are also difficult to resolve in our infection model, as we find that virus ruptures membranes much earlier in the endocytic cycle than the necessary drop in endosomal pH to prime hexon would occur. However, they do show that expressing hexon in HeLa cells 48 hours prior to viral





**Figure 21: Contributions of hexon HVR1 in dynein interactions.** HeLa cells were transduced with the indicated virus in the presence or absence of 50 $\mu$ M Ciliod. GFP-positive cells were assessed by flow cytometry. Infectivities are normalized relative to DMSO vehicle controls.

infection restricts Ad accumulation at the nucleus one hour post-entry [290], indirectly indicating a role for hexon in viral transport. Interestingly, they also point out that hexon overexpression disorganizes microtubules at the periphery, but do not explore this further. Taking into consideration our DLC1 siRNA (Figure 9) and Ciliobrevin D (Figure 10) results in conjunction with the Ad5-HVR1D3 data, these observations would indicate that, while dynein is clearly important in Ad entry, virion interactions do not occur through DLC1 or Ad5 hexon HVR1.

These results suggest a complex interplay in viral capsid proteins during transport. Our Ad5-M1 data plainly demonstrate that the pVI-PPxY motif is a critical component in viral exploitation of dynein for endosomal egress, but do not specify whether dynein-virus interactions downstream of egress occur, and if so certainly do not indicate what proteins are involved in such an interaction. It is possible that, although hexon likely does not interact with dynein in a pH-dependent manner, it still has the capacity to interact with some subunit of dynein in the context of virus entry after partial disassembly, once the virion egresses from the endosome into the cytosol. Other capsid proteins that are associated with the penetrating virion prior to genome delivery (proteins V, VII, and X) do not interact with the dynein 74.1 kDa intermediate chain or the dynactin component p150<sup>Glued</sup>, and do not affect viral targeting to the nucleus during entry, suggesting they are not involved in microtubule-mediated viral transport [290]. Fiber, penton base, pIX, pIIIa, and pVIII were not assessed, as they were presumed lost upon uncoating. It is important to fully characterize which viral proteins interact with dynein *in vivo* during infection, and at the discrete steps of entry these interactions occur, and designing tightly

controlled experiments will help to elucidate these steps. Using small molecule inhibitors such as ciliobrevin D to regulate dynein function on a small time scale with putative dynein-interacting Ad mutants will further aid in delineating the mechanisms of dynein recruitment both upon endosomal rupture and after viral penetration into the cytosol.

### *Kinesin*

Kinesin function, while also implicated in Ad entry, has not been studied in as much detail as dynein interactions. Although bi-directional movement is observed in live cell imaging of Ad during entry, microinjecting antibodies against kinesins has no effect on virion accumulation at the nucleus [289]. This, in combination with data suggesting that fewer kinesin molecules interact with the capsid compared to dynein [389] likely led to the assumption that kinesin is dispensable for efficient virus infection. However, a recent publication indicated that cells knocked down for KLC1 were defective for Ad genome delivery compared to control cells [291]. Their data suggest that pIX interacts with kinesin and the NPC at the nuclear membrane to mediate NPC disruption and genome delivery into the nucleus. However, although pIX interacts with KLC1 in *in vitro* pull down assays, we found that knocking down KLC1 similarly restricts both Ad5-WT and Ad5- $\Delta$ pIX viral infectivities (Figure 19). These data, along with our other results, indicate to us that a) kinesin knock down inhibits Ad infection independently of pIX present on the capsid, and b) the defect in Ad5- $\Delta$ pIX lies in endosomal egress downstream of evading autophagy. However, how kinesin affects virus infection still remains a valid question.

Since pIX is not involved in kinesin-facilitated viral infection, this suggests some other capsid protein is involved. During their initial assessments, Strunze, *et al.* found that pIIIa and small quantities of fiber are also pulled down by KLC1 TPR after heat-dissociating virions [291]. They dismissed these as putative kinesin interactors since they are quickly shed from the capsid during entry. However, it may be wise to assess these capsid proteins for kinesin interactions in immunoprecipitation assays, and further determine kinesin-binding residues if they do interact. Repeating these infectivity studies with mutant viruses that do not interact with kinesin via these proteins might allow us to determine how kinesin aids viral infection.

Further attempts to assess the defect in Ad entry caused by kinesin knock down suggested are preliminary. We found that, although Ad5-WT is internalized and ruptures endosomes equally efficiently in both control and KLC1 siRNA cells, there are fewer virions associated with gal3-positive endosomes at 30 minutes in kinesin knock down cells when compared to control (Figure 19). Furthermore, knocking down kinesin does not appear to affect Ad5-WT accumulation at the nucleus, corroborating previously published reports (data not shown). How kinesin apparently impedes early viral endosomal escape but facilitates efficient infection requires further investigation. The report from the Greber lab suggests that knocking down kinesin prevents proper uncoating of the virus at 3hpi. Taken together, these results may indicate that, although virus enters into the cytoplasm faster in the absence of kinesin, relevant proteins within the viral capsid are not in the proper conformation due to inadequate uncoating. Perhaps a mechanical “tug-of-war” on the capsid between kinesin and another protein (such as

dynein or membrane fragment-bound receptors) facilitate appropriate capsid dismantling prior to endosomal egress. Testing this would first require determining the capsid proteins that physiologically interact with kinesin during entry, and further defining the precise sequences that specifically interact with the motor. Once these sequences are known, Ad5 mutants could be generated that contain a scrambled sequence that does not bind to kinesin. If kinesin-protein interactions facilitate virus uncoating, then fewer virions would disassemble in cells transduced with the mutant virus compared to WT. We would expect that the mutant would also egress from endosomes with kinetics similar to what we observed in KLC1 siRNA cells.

When it is not transporting molecules throughout the cell, the kinesin-1 complex tail folds in on the motor head and exists in an autoinhibited conformation [390]. In *Drosophila*, binding synthetic cargo releases kinesin from this state, allowing transport on microtubules [390]. However, research utilizing mammalian kinesin-1 suggest that binding native cargo alone may not sufficiently relieve kinesin from autoinhibition [391]. More recently, it was found that the RAN-binding protein 2 (RANBP2) is an allosteric activator of kinesin-1 [109]. RANBP2, also known as Nup358, is a major nucleoporin found at the NPC, and previous data indicate that although Nup358 knockdown does not restrict Ad targeting to the nucleus, it inhibits Ad uncoating similar to the observations found with KLC1 siRNA cells [291, 392]. It would be interesting to assess whether knocking down both KLC1 and Nup358 further restricts viral infection compared to individual knock downs. Perhaps Nup358 must activate virion-associated kinesin to both facilitate proper uncoating and simultaneously give the virus the opportunity to egress

from ruptured endosomes. A previously published model postulates that this event also supports genome delivery, but this hypothesis was not tested in either their hands or ours [291]. Since many studies suggest that genome delivery occurs with membrane-free virions, we hypothesize that virus egressing from lysed, nuclear-targeted endosomes must traffic back to the nucleus at a later time to deliver their genome. If this is the case, it would be important to further determine whether capsid dissociation occurs upon viral penetration into the cytoplasm or after viral relocalization to the NPC.

### **Protein IX function in Ad entry**

To understand the intricacies of Ad infection, it is important to define the contributions of the protein components of the viral capsid in terms of their interactions with the host. In revealing these interactions, we will further delineate potential influences of the capsid proteins both in aiding viral entry and their effects in evading detection or restriction. In this study, we focus on the contributions of a so-called minor capsid protein, pIX, during entry. Until recently, capsid-associated pIX was thought to only act as a capsid stability protein, as assessed by increased capsid thermolability in virions deleted for the protein. A recent publication implicates pIX involvement in interacting with kinesin, but we show here that kinesin's contributions to Ad infection are independent of the presence of pIX. Moreover, we show that Ad5- $\Delta$ pIX's defect in infectivity is a result of an inability of virions to properly penetrate into the cytosol.

To further characterize pIX's activity during viral entry, we attempted to assess the contributions of individual domains of the protein. The protein consists of three putative domains: an N-terminal domain (NTD), a C-terminal domain (CTD), and an

alanine-rich linker [205]. The NTD is critical for incorporation into the viral capsid, and embeds into hexon trimer interfaces within each facet of the capsid [305]. Immunogold labeling reveals that the NTD in mature capsids is not detectable by antibodies [393]. Conversely, the CTD is not required for incorporation, but is recognized by antibodies in both intact and disrupted virions, suggesting it is exposed to the cytoplasm during entry. Thus, we expected to find that the pIX CTD is critical in assisting viral infection. To test this, we stably expressed either HA-tagged full-length pIX (HA-pIX-FL) or protein that is truncated for its CTD (HA-pIX $\Delta$ CTD) in HeLa cells. When we transduced Ad5-WT or Ad5- $\Delta$ pIX, we observed enhanced infectivities of both viruses in HA-pIX-FL compared to control cells (Figure 18).

This result surprised us, since we expected that soluble HA-pIX in the cells would act as a competitive inhibitor to restrict WT infectivity. However, previous reports show that pIX expression in cells either through viral transduction or transfection produces pIX-positive inclusion bodies in host cell nuclei [305, 309]. These inclusion bodies sequester promyelocytic leukemia protein (PML), and during normal viral replication this event is important for optimal virion production. Nonetheless, even if the majority of HA-pIX is found within the nucleus, some protein should be present in the cytoplasm to compete with Ad5-WT for pIX-interacting partners during transduction. Perhaps pIX stably expressed in cells constitutively sequesters PML bodies, significantly enhancing virus infection greater than what is accounted for in the Ad5- $\Delta$ pIX virus' defect. However these data do not exclude the possibility that pIX-host interactions are important for efficient Ad escape from endosomes during entry. To separate these functions, it may

be possible to design a pIX mutant that does not import into the nucleus. This way, pIX should remain in the cytoplasm to interact with host proteins, but cannot inhibit PML activity. We would then anticipate a restriction in Ad5-WT infectivity in cells expressing the pIX mutant compared to control cells. Further, there should be no change in Ad5- $\Delta$ pIX infectivity.

IFA studies assessing Ad5-WT colocalization with gal3 in HA-pIX stable cells may also provide insight into whether HA-pIX actually acts as a dominant negative for Ad endosomal escape. If it has dominant negative activity, we would expect to see more Ad5-WT colocalize with gal3 compared to controls. The results from the IFA experiments, together with the transduction assay data, could distinguish two distinct roles pIX has during adenovirus entry: one in properly targeting virions to NPCs, and another in inhibiting PML body activity at the early stages of replication. Interestingly, transducing cells stably expressing HA- pIX $\Delta$ CTD yields a slight, but significant, increase in both virus' infectivity. Previous literature indicates that the C-terminal coiled-coil domain is important for pIX trans-activating and PML sequestering activity. Our data indicate that while this likely is predominantly the case, the N-terminal or linker domains of pIX may play a part in facilitating Ad infectivity. Again, ongoing IFA studies will help to elucidate which of these domains are important for mediating virus escape from endosomes during entry.

How stably expressing pIX in cells enhances infection is unclear. A confounding observation in a previous study shows infection with Ad5- $\Delta$ pIX does not disrupt NPCs as well as WT Ad, as assessed by 40kDa dextran-FITC infiltration [291]. Although capsid-

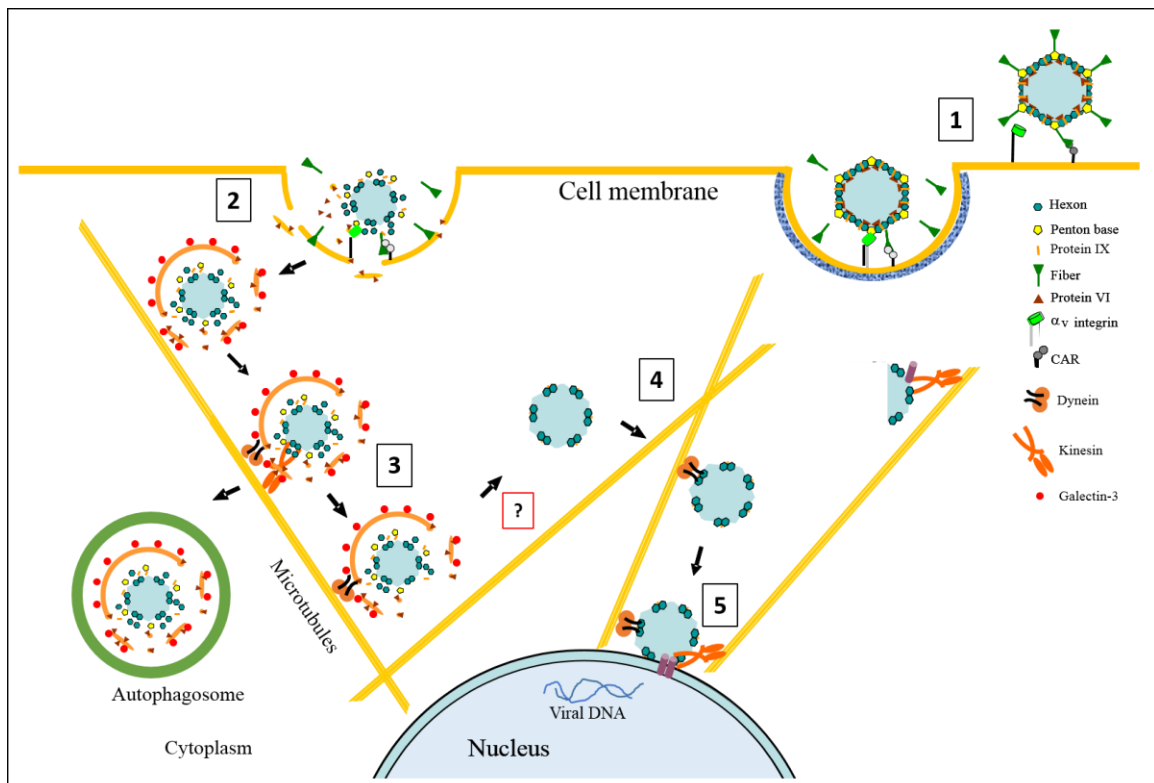


associated pIX is important for NPC disruption, it is not known whether pIX plays a direct role in the event. Similar to these observations, NPC disruption is also prevented in WT infections when KLC1 or Nup358 are knocked down by siRNA [291]. These data explicitly implicate a role for all of these molecules at the NPC, but how they are involved with each other is still not understood. We find that KLC1 knock down restricts Ad5- $\Delta$ pIX virus as effectively as Ad5-WT, suggesting that these defects are distinct from each other. Whether the deficiencies in infection observed with KLC1 and Nup358 knock downs are part of the same event has not been assessed.

Although virus deleted for pIX more or less properly traffics to the perinuclear area, it does not colocalize with Nup358/Nup214 NPC complexes as efficiently as WT. Strunze, *et al.* do show that pIX-deleted virus still interacts with the KLC1 TPR, just not to the extent observed with WT virus [291]. The little amount of kinesin still present may be enough for the motor to enact its function during infection. This could explain why knocking down KLC1 inhibits both Ad5-WT and Ad5- $\Delta$ pIX virus, and these results may suggest that the mutant virus' failure to disrupt the integrity of the NPC is simply due to an incapacity to accurately target to NPCs. If Nup358 is important to activate kinesin associated on the viral capsid as described above, then this would also explain why Ad5- $\Delta$ pIX is retained in endosomes at the nuclear membrane. This could be tested by assessing WT colocalization with gal3 in Nup358 knock down cells. If viral targeting to Nup358 is important for viral egress, then WT virus will be retained in gal3-positive endosomes at the nucleus in knock down cells compared to control cells.

Taking all of our data together, we hypothesize a two-step model of viral accumulation at the nucleus (Figure 22). After membrane rupture at or near the cell surface, gal8 is recruited by luminal N-linked glycans to alert the host to danger. Autophagy machinery is quickly recruited to sites of injury, as evidenced by our observations showing that both gal3 and LC3 puncta are induced and colocalize with virus within 30 minutes of virus internalization [324]. LC3 cleavage and aggregation at sites of damage during Ad infection is dependent on TBK1 activity (Figure 4), suggesting a role for the kinase in autophagosome nucleation and/or expansion. Simultaneously, the ruptured endosome recruits microtubules and their associated proteins via the PPxY motif within protein VI. Multiple results demonstrate that pVI-M1 is defective for microtubule associations: transient transfections of pVI-M1 do not colocalize with microtubules as well as pVI-WT, live cell imaging suggests that pVI-WT traverses the cytoplasm bidirectionally and with high velocities whereas pVI-M1 does not do so as efficiently [209], and treating cells with nocodazole to depolymerize microtubules does not restrict Ad5-M1 infectivity as severely as Ad5-WT [324].

Our ciliobrevin D results indicate that after microtubules are recruited, dynein interacts with membrane fragment-associated virus and transports it towards the nucleus, since virions in CilioD-treated cells are confined within gal3-positive endosomes at the cell periphery (Figure 10). Whether the virus takes advantage of dynein to evade autophagy machinery remains unclear, since autophagosome formation and lysosomal targeting also require dynein. The PPxY motif likely targets virus confined within endosomes away from sites of expanding, but incomplete, autophagosomes to mediate



**Figure 22: Two-step model of adenovirus entry.** (1) Adenovirus interacts with primary (CAR) and secondary ( $\alpha_v$  integrin) cell surface receptors, inducing virion uptake via clathrin-mediated endocytosis. (2) Mechanical perturbations between the receptors and virus partially dissociate the capsid, revealing pVI. Freed pVI intercalates into the lipid bilayer and ruptures the endocytic membrane at or very near the cell surface, recruiting galectins to initiate the host danger response and activate autophagy machinery. (3) Endosome-associated virus engages microtubules via dynein, translocating to perinuclear locations. Virus egresses from endosome fragments towards the cell periphery by unknown mechanisms, where (4) it again interacts with microtubules to target to NPCs. (5) Opposing forces between virus, microtubule motors, and nucleoporins disrupts the NPC and the viral capsid, importing the viral genome into the nucleus to initiate replication and the generation of progeny virions.

virion evasion of lysosomal degradation. Regardless, dynein is necessary to mobilize virus-endosome complexes to the perinuclear area. Additionally, our IFA data in KLC1 knock down cells imply that KLC1 interactions may be important to retain virus within endosomes during cytosolic transport, potentially until the capsid reaches an area where it can properly uncoat (Figure 19).

Once virus is perinuclear, our live cell imaging assays show that the virus egresses from gal3-positive endosomes towards the cell periphery. Three observations suggest that virus escaping from endosomes at the nucleus is likely not delivering its genome into the nucleus. First, previous reports indicate that virus targeting to NPCs are free of endosomal membranes [291, 394, 395]. Second, we show here the Ad5- $\Delta$ pIX is retained in perinuclear gal3-positive vesicles (Figure 17), and third, Strunze, *et al.* report that Ad5- $\Delta$ pIX is not targeted to NPCs on the nuclear membrane [291]. Taken together, these data suggest that virus penetrates into the cytosol at the nuclear periphery, and moves towards the cell periphery. The membrane-free virus must then traffic back to the nucleus and target to Nup358/Nup214-positive NPCs to deliver its genome. Others suggest that dynein interacts with the HVR1 region of hexon to traffic naked virus toward the nucleus so the capsid can dock at the NPC [290, 340, 396]. However, as described earlier in this text they suggest that hexon requires priming by pH 4.4 to facilitate such an interaction, and we and others previously showed that Ad5 ruptures endosomes well before they achieve a pH that low. As such, it is unclear how the virus mediates transport back to the nucleus to dock at the NPC. Regardless, once at the NPC, dynein becomes anchored to Nup214, and kinesin interacts with nup358 and capsid. Opposing motor

functions between dynein and kinesin disrupt the NPC and fully uncoat the capsid, revealing the viral genome and facilitating its delivery into the nucleus [291].

### **Contributions of other factors during Ad Infection**

There are other factors, both viral and host, not focused on in this study that are important during infection. How these varied components influence each other to affect viral entry require broader study. Recent studies on some of these factors may shed light on such influences.

#### *Calcium influx*

A very recent report shows that Ad-induced membrane damage at the plasma membrane mediates calcium influx into cells [397]. The study suggest that increases in calcium concentrations in the cell facilitate lysosomal exocytosis, increasing ceramide concentrations at the plasma membrane. In turn, this activity leads to increased endocytosis of membrane lesions, which expedites viral uptake. Additionally, other reports indicate that mitochondria traffic to area with high concentrations of calcium in neurons via dynein- and kinesin-dependent transport [398, 399]. Once present at these sites, the high calcium concentrations arrest kinesin motor activity, which is only relieved once calcium levels drop. Perhaps calcium influx directs dynein and kinesin to points of viral endocytosis, where the motor proteins can interact with the virion immediately upon vesicle rupture to facilitate the evasion of autophagy machinery. In this model, kinesin activity is hindered until the virus traffics away from sites of autophagosomal formation, allowing dynein motor function to dominate. This model would implicate a mechanism of microtubule motor recruitment to Ad-containing endosomes.

### *Adenoviral protease*

The adenoviral protease (AVP) has a very well characterized role in viral replication during the capsid maturation process, whereby cleaving viral preproteins within the formed capsid puts the virion in a metastable conformation to facilitate disassembly upon infecting a new host cell [189]. The mature Ad5 virion contains 11 copies of AVP [191], however due to its essential function in replication, its importance during entry is difficult to characterize. Chemical inhibition of the protease via reduction and alkylation/oxidation of virions prior to transduction severely inhibits viral infection by approximately 80% at the disassembly stage, after virus targets to NPCs [191]. Although the protease is the major apparent target of NEM-mediated alkylation, penton base, fiber, pV, and two unidentified proteins are also alkylated in their inactivation procedure. Thus, the contributions of AVP in viral entry relative to these other proteins is not entirely clear. Interestingly, pVI degradation is inhibited in NEM-alkylated virus preparations, yet pVI itself contains no cysteine residues for NEM to alkylate. Although membrane rupture is not apparently affected upon alkylation, whether another alkylated viral protein contributes to pVI function during viral entry, and at what step this may occur requires further testing. Subsequent publications suggested that E1A inhibits nitric oxide synthase, and nitric oxide itself restricts protease activity *in vitro* [400, 401].

We attempted to assess the explicit contributions of AVP during entry using newly developed AVP inhibitors, 37248 and 37249 [402]. These inhibitors restrict AVP cleavage function 9- (37248) and 35-fold (37249) *in vitro*, but were not tested in cell culture conditions. We tested the effect of these inhibitors on Ad5-luc infectivity, with

VSVg-luc as a control for inhibitor activity. Although we found these inhibitors restrict luc production in cells transduced with Ad5-luc, they also similarly restrict VSVg-luc infectivity (data not shown). These results suggest to us that, in addition to their effects on AVP, these inhibitors also affect the activity of one or more host factors. Greber, *et al.* showed that AVP alkylation only occurs after the virion is treated with the reducing agent DTT, suggesting that the chemical can only access the protease upon reduction of the virion. More intricate studies that specifically target the AVP without affecting other viral or host protein activities need to be designed to precisely elucidate its specific contributions.

#### *Host-mediated viral ubiquitination*

Although Nedd4.2 mediates pVI ubiquitination in *in vitro* assays, the physiological effects of this remain untested. Furthermore, it is not known whether other Ad5 capsid components are ubiquitinated during entry. Pathogen ubiquitination is a critical step in targeting intracellular bacteria for autophagy, implying that a similar process could occur during Ad entry. Determining the proteins that are ubiquitinated after membrane rupture would aid in determining what components of the capsid and/or host mediate the targeting of autophagy machinery to sites of endosomal damage. Furthermore, it would potentially provide additional targets to assess for infectivity defects during entry.

Leucine-rich repeat and sterile alpha containing motif 1 (LRSAM1) and Parkin are E3 ubiquitin ligases recently described to respectively ubiquitinate *Salmonella typhimurium* and *Mycobacterium tuberculosis* organisms upon membrane rupture [74,

403]. In the case of *Salmonella* infection, LRSAM1 did not fully inhibit pathogen ubiquitination, implicating other unidentified ubiquitin ligases are also involved. These data imply that Nedd4.2 may not be the only ubiquitin ligase involved in Ad infection. Identifying if this is the case, and further teasing out the contributions of each ligase during different pathogen infections, may aid in our understanding of the differential regulation in autophagy targeting by the host. Both LRSAM1 and pIX contain leucine-rich repeat (LRR) domains, which are thought to act as protein recognition motifs [305, 404, 405]. Whether the LRR domain of either protein is important for protein interactions during Ad entry is unknown. Interestingly, observations suggest that LRSAM1 may stabilize NDP52 associations on ubiquitinated pathogens [74]. Whether such stabilization is required to target autophagy machinery, or if it is only explicitly important during *Salmonella* infections, is unknown. Assessing whether NDP52 still targets to Ad-ruptured endosomes in cells knocked down for LRSAM1 would be a first step in determining the role of LRSAM1 in autophagy.

Previous reports show that AVP contains deubiquitinating (DUB) activity late in infection between 24 and 36 hpi [192]. DUB activity is enriched in acid soluble nuclear fractions, and specifically deubiquitinates mono- and di-ubiquitinated histone H2A. No studies have assessed whether AVP has global DUB activity at early times during replication, but others found that Ad does not appear to induce global ubiquitination of host or viral proteins as detected by western blot ([406], personal communication from Harald Wodrich). More specifically, no data establish whether AVP enacts local DUB activity during entry. It would be interesting to assess whether AVP DUB activity is



important for viral entry, but as stated above it is difficult to manipulate AVP activity in entry without affecting its essential functions in replication. If possible, separating the AVP deubiquitinating activity from its viral proteolysis function may aid in assessing whether it plays a role in viral entry.

*Consequences of microtubule post-translational modifications during Ad entry*

Although MTs have such a significant role in cell function and viral manipulation, there is still a vast gap in our knowledge in how MTs specifically interact with and transport cargo. We are only just beginning to understand how MT PTMs affect cargo specificity and protein interactions, but many studies are still necessary to fully comprehend the contributions of individual PTMs. Ads require MTs for transport within the cell and ultimately to the nucleus, to deliver their viral genome. Others previously showed that MT stabilization with paclitaxel during Ad transduction enhances the nuclear accumulation of virions [407]. Furthermore, Ad transduction enhances MT stabilization via PTMs within one hour post-entry [408]. This report also notes that Ad infection enhances MT growth after nocodazole washout, compared to mock infected cells. MT stabilization in Ad infections is much faster compared to other viral infections, and occurs via increased acetylation and detyrosination of tubulin, shifting the balance of MT dynamics towards a state of net growth. Ad mediates MT PTMs by the Rho GTPases RhoA and Rac1. Whether microtubule stabilization via acetylation specifically enhances Ad transport, or is necessary to recruit particular MAPs is not known.

The eukaryotic deacetylase HDAC6 is implicated in deacetylating tubulin; overexpression of HDAC6 induces a global decrease in acetylated  $\alpha$ -tubulin, whereas

HDAC6 depletion increases acetylated  $\alpha$ -tubulin pools. Perhaps HDAC6 expression could be utilized to assess the effects of tubulin acetylation on Ad entry. Ad localization in cells either overexpressing or knocked down for HDAC6 could be assessed by IFA. If MT acetylation is important for proper Ad targeting, then overexpressing HDAC6 should restrict Ad accumulation at the nucleus, and HDAC6 depletion may enhance virion transport to the perinuclear area. If this is the case, further assessments to determine whether virus still escapes endosomes in the absence of MT acetylation can be performed. Although reports using Ad to probe microtubule PTMs are sparse, researchers are now beginning to focus efforts on this area of investigation. Studies using Ad will allow researchers to flesh out the molecules involved in MT PTMs during viral infection, and global effects enacted on the host cell under these conditions.

#### *Intermediate filaments*

In addition to microtubules, the cell's cytoskeleton is also composed of actin and intermediate filaments (IFs). Actin is required for initial Ad virion endocytosis, and actin translation is later shut down during replication [251, 409]. Compared to microtubules and actin, however, the function of IFs during Ad entry are not well characterized. Cytokeratin 18, an IF found in epithelial cells, is cleaved by AVP late in infection, contributing to cytopathic effect (CPE) and viral release [410, 411], however its integrity is maintained normally during Ad entry. Conversely, another IF called vimentin is quickly depolymerized by the host during entry – within five minutes – but has no effect on CPE [412]. Vimentin is important both in cell integrity and in securing the positions of various organelles within the cell, including the nucleus, ER, and mitochondria [413,

414]. Moreover, Ad interacts with both tubulin and vimentin 30 minutes after infection [415], suggesting that vimentin may have some function during Ad entry. Unfortunately, this role is unclear as further studies of vimentin using Ad were not pursued. Nevertheless, vimentin studies in other viral infection models implicate its necessity during infection.

Both parvovirus and foot-and-mouth disease virus replication requires a functioning, intact vimentin structure in host cells [63, 416]. Furthermore, studies using African swine fever virus show viral factories in infected cells require the formation of a vimentin “cage” around them for optimal replication [417]. In this model, vimentin is phosphorylated by CaM kinase II, but it is unknown whether this phosphorylation event is required for the caging phenomenon. Notably, beclin-1 interactions with vimentin via regulatory 14-3-3 proteins inhibits autophagosome formation [418]. Perhaps vimentin is processed during Ad infection to enhance the activation of autophagy. We attempted to assess whether acrylamide-inhibition of vimentin affects Ad infectivity, but results were inconsistent and not pursued further (data not shown). Still, it could prove useful for future studies to assess if vimentin interactions with the viral capsid has a functional role during Ad entry. Uncovering such contributions, if any, as well as determining which capsid proteins enable vimentin binding may provide insight as to how Ad might exploit the IF arm of the cytoskeleton.

## **Adenovirus Mutants as Potential Vaccine Vectors**

### *Introducing mutations into less seroprevalent Ad species*

As stated earlier, Ads are useful base vectors for designing vaccine platforms. Using the various defects of the mutants we have studied, we can further specifically target these vaccine vectors to various locations within the cell for their designated function. For example, we can utilize the Ad5-M1 mutant for vectors that contain pathogen epitopes on the viral capsid to enhance their delivery to lysosomes. Lysosomal targeting degrades the virus, and increases epitope loading onto MHC class II for CD4<sup>+</sup> T cell recognition. Although we utilize Ad serotype 5 for our studies in cell culture, it is not an optimal candidate for Ad vaccine vectors due to very high levels of preexisting immunity worldwide [419]. Previous studies suggest that neutralizing antibodies against Ad mainly target the hypervariable region of hexon [420], and current research is aimed at replacing these regions in recombinant Ad5 backbone vectors to circumvent immunity [421, 422]. While early tests are promising, concurrent studies utilizing other, less seroprevalent Ads may also prove fruitful.

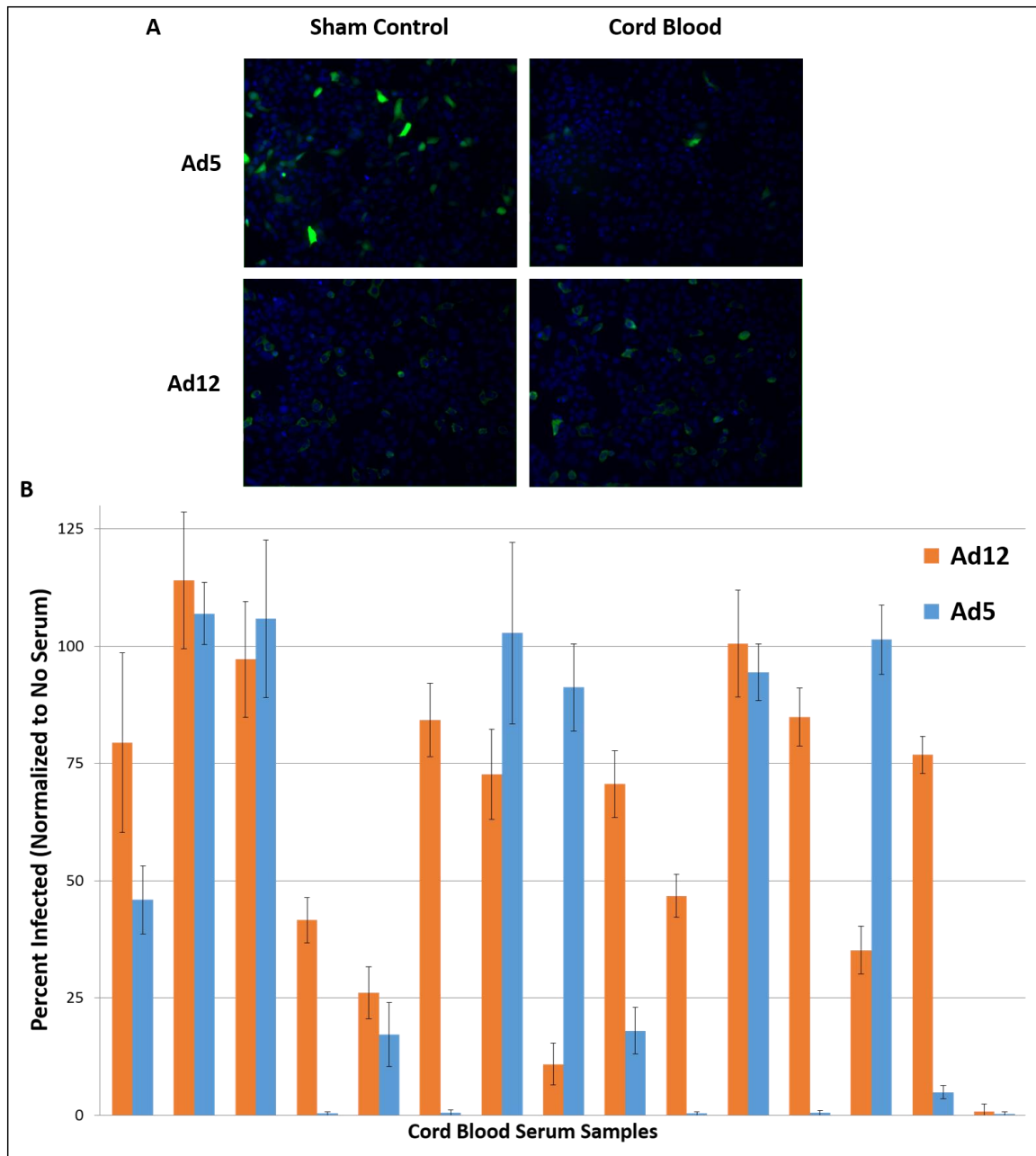
Ad12, a subgroup A virus, is one of the more phylogenetically distinct human Ads compared to Ad5 [423, 424]. Replacing the Ad5 hexon HVR with Ad12's sequence produces a viable chimeric virus capable of bypassing Ad5 preexisting immunity in mice. We compared the seroprevalence of neutralizing antibodies against Ad12 and Ad5, and found that Ad12 seroprevalence in local cord blood samples is significantly lower compared to Ad5 (Figure 23). Nine of the fifteen samples (60%) inhibit Ad5 infectivity by greater than 50%, and eight are restricted by more than 75% (53% of samples).

Conversely, only 6 cord blood samples (40%) inhibit Ad12 infectivity by 50%, and only 2 (13%) restrict Ad12 more than 75%. Furthermore, Ad12 infectivity is 100-200-fold defective compared to Ad5 in culture, and Ad12-infected dendritic cells induce CD4<sup>+</sup> T cells to secrete more IFN $\gamma$  compared to Ad5-WT, and at similar levels compared to Ad5-M1 (Figure 24). Since both Ad5 and Ad12 pVI contain a conserved PPxY motif, it would be interesting to assess whether introducing the M1 mutation into Ad12 would further enhance antigen presentation and CD4<sup>+</sup> T cell-mediated immune responses. These studies, along with additional research using Ad12 and other rare Ad serotypes, could prove fruitful in attempting to develop more targeted Ad-based vaccine vectors.

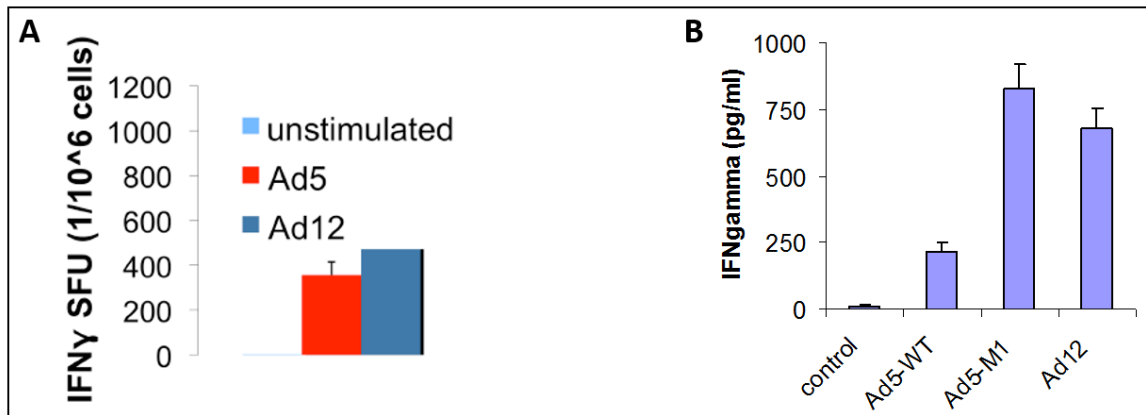
## Conclusions

Overall, in this study we identify the mechanism of host sequestration of Ad-mediated damaged endosomes through autophagy occurs via TBK1 activity. TBK1 facilitates LC3 cleavage, likely to expedite autophagosome formation. Although the signaling cascade to induce autophagy was not ascertained, we eliminated some potential pathways mediated through ROS signaling. We also explicitly determined the contributions of dynein in Ad evasion of autophagy. Chemical inhibition of dynein motor function restricts Ad absconding from ruptured endosomes and reduces nuclear targeting of virions. Furthermore, we revisited previous reports implicating that kinesin recruitment to Ad capsids is mediated by pIX. We found that kinesin depletion restricts virus infectivity in a pIX-independent manner, contradicting the previous findings. More detailed studies to assess the defect suggest that kinesin knock down enhances viral egress at very early times. Data from other labs indicates that virions in cells knocked

down for kinesin do not efficiently uncoat, suggesting a role for kinesin during endosomal egress properly primes the virion to properly dissociate and deliver its viral genome. Future studies will specifically determine the viral proteins interacting with kinesin, and how such interactions facilitate uncoating.



**Figure 23: Ad12 pre-existing immunity.** HeLa cells were incubated with cord blood diluted with DMEM to 1:16 its concentration and transduced with Ad5gfp or Ad12. Ad12 samples were stained with rabbit anti-Ad12 antisera and 488-conjugated goat anti-rabbit secondary antibody. Cells were imaged and quantified by hand using ImageJ software **A.** Representative images of cells transduced with Ad5gfp or Ad12 in the presence of cord blood or DMEM alone. **B.** Effects of 15 different cord blood samples on Ad5 and Ad12 infectivity. Percent infections are normalized to DMEM only sham infections, standardized to 100%. Pre-existing immunity was determined as inhibiting infection by greater than 50% relative to controls.



**Figure 24: CD4<sup>+</sup> T cell activation by Ad vectors.** **A.** Splenocytes from Ad5-infected mice were stimulated ex vivo with replication-defective Ad5 or UV-inactivated Ad12. IFN $\gamma$ -producing cells determined by ELISPOT. **B.** Human monocyte-derived DCs were infected with the indicated virus for 2 hours. Cells were washed and incubated with an equivalent number human CD4<sup>+</sup> T cell clones recognizing a conserved peptide in hexon (amino acids 910-924 in Ad5) for 24 hours. Supernatants were analyzed for IFN $\gamma$  by ELISA.



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## VITA

Andrew Burrage was born in Tampa, Florida on September 13, 1986 to Michael Burrage and Mindy Myre. He attended the University of Florida in Gainesville, FL for his undergraduate studies from 2005-2010. There, he received a Bachelor of Science with a major in chemistry and a minor in business administration. While at UF, Andrew was an undergraduate research assistant in the laboratory of Dr. Richard Moyer, where he studied poxvirus infections in animal models under the guidance of Dr. Amanda Rice.

Andrew joined Loyola University Chicago's Microbiology and Immunology Department at the school's medical campus in Maywood, IL in 2010, where he joined the laboratory of Dr. Christopher M. Wiethoff. There, he studied adenovirus immune evasion during cell entry, with a focus on viral evasion of autophagy and exploitation of microtubules. Andrew is a Loyola Research Mentoring Program awardee, and received a fellowship award from the Schmitt Foundation to fund his last year of graduate studies.

In October 2015, Andrew married Dr. Sara Jones, a postdoc in Dr. Katherine Knight's laboratory. After completing his Ph.D., Andrew will begin a post-doctoral position in the laboratory of Dr. Dan Kearns at Indiana University in Bloomington, IN. There, he will study bacteriophage function in the bacterium *Bacillus subtilis*, as well as the roles of signaling molecules involved in motility and swarming switching within the organism.